THE SPECIFICITY OF LEUCINE AMINOPEPTIDASE*

BY EMIL L. SMITH AND N. BALFOUR SLONIM†

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the Departments of Medicine and Biological Chemistry, University of Utah School of Medicine, Salt Lake City)

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Since Linderström-Lang’s demonstration that the hydrolysis of L-leucylglycine (LG) is due to a distinct leucyl peptidase (1), various studies have shown that this enzyme is widely distributed (2–4) and requires for its activity the presence of Mn⁺⁺ or Mg⁺⁺ ions (2, 5). The enzyme has been regarded as a typical aminopeptidase (5), since it does not hydrolyze acylated compounds such as benzoyl-L-leucylglycine, and since, in addition to the dipeptide, it hydrolyzes L-leucinamide (LA) and the tripeptides, L-leucylglycylglycine (LGG) and L-leucyl-L-leucylglycine.

It has now been observed that a highly purified preparation of leucine aminopeptidase from hog intestinal mucosa can hydrolyze glycyl-L-leucinamide (GLA). Under the conditions of our experiments, the reaction ceases after the decomposition of a single peptide bond. Since glycyl-L-leucine is not appreciably hydrolyzed, the hydrolysis must occur at the terminal amide bond, as indicated by the dotted line; R represents the isobutyl side chain. Thus, the products of the reaction must be glycyl-L-leucine and ammonia. If the action occurred at the other peptide bond, liberating glycine and LA, the second peptide bond would also be split, since LA is rapidly hydrolyzed by the enzyme. The observation that GLA is hydrolyzed by leucine aminopeptidase has been possible only because the purification process has removed the glycyl-L-leucine dipeptidase. With crude extracts of hog intestinal mucosa and other tissues, the consecutive hydrolysis of both peptide bonds occurs (6).

Table I shows that the hydrolysis of GLA follows the kinetics of a first

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### Table I

**Specificity of Leucine Aminopeptidase**

The enzyme was incubated at 40° in veronal buffer at pH 7.8 to 8.0 with 0.01 M Mn++, for 3 hours prior to addition to the buffered substrate solution (0.05 M). The proteolytic coefficient $C = K/E$, where $K$ is the first order velocity constant for the enzyme concentration $E$ expressed in mg. of protein N per cc. of test solution. Two different enzyme preparations of somewhat different activities were used; these are distinguished by the letters in parentheses following the substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme concentration (mg. protein N per cc.)</th>
<th>Time (hrs.)</th>
<th>Hydrolysis (%)</th>
<th>$C$</th>
<th>$C$ average</th>
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</thead>
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<tr>
<td>$\text{L-Leucinamide (a)}$</td>
<td>0.8</td>
<td>0.5</td>
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<td>3.7</td>
<td>3.9</td>
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<td></td>
<td></td>
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<td>4.1</td>
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<td>1.5</td>
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<td></td>
<td>2.0</td>
<td>58</td>
<td>4.0</td>
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<td>2.5</td>
<td>66</td>
<td>3.9</td>
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<td>$\text{Glycyl-L-leucinamide (a)}$</td>
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<td>0.5</td>
<td>13</td>
<td>2.5</td>
<td>2.2</td>
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<td></td>
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<tr>
<td>$\text{L-Leucinamide (b)}$</td>
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<td>$\text{Glycyl-L-leucinamide (b)}$</td>
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<td>$\text{Carbobenzyox-L-leucinamide (b)}$</td>
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<td>20</td>
<td>0</td>
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<td></td>
</tr>
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</table>

* Used at 0.1 M. We are indebted to Dr. J. S. Fruton for a sample of this compound.

The hydrolysis of all the sensitive compounds is strongly activated by Mn++, and the hydrolysis of LGG follows the kinetics of a zero order reaction. The approximate initial first order constant is given for comparison with the other substrates.
activation of the enzyme by Mn++ follows the same type of time reaction
for GLA as for the other substrates (5).

Because of our finding that leucine aminopeptidase hydrolyzes an amide
linkage at a distance from the free amino group, as in GLA, the possibility
that the enzyme may possess some endopeptidase activity was investigated.
Fruton and Bergmann (7) have reported that chymotrypsin shows a dual
specificity and can act on substrates characteristic both of endopeptidase
and aminopeptidase specificities. We have, therefore, tested the action
of our enzyme on a variety of substrates. These results are also presented
in Table I.

It is clear that leucine aminopeptidase does not have any detectable
endopeptidase action, since no hydrolysis could be observed with carbo-
benzoxycarbonylglycyl-L-leucinamide, carbobenzyloxycarbonylglycyglycyl-L-leucinamide, car-
obenzyloxycarbonylglutamyl-L-leucinamide, or other N-acylated peptides. On
the other hand, compounds with a free amino group such as L-leucyl-L-
glutamic acid and L-glutamyl-L-leucinamide (GLA) are readily hydrolyzed.
With GLA only one peptide bond is hydrolyzed when the purified enzyme
is used. However, with a crude extract of intestinal mucosa, both bonds
are rapidly hydrolyzed.

It is of interest that the enzyme preparation has a slow but definite action
on glycylglycyl-n-leucylglycine (GGLG). One must assume that this
action is due to the leucine aminopeptidase, since no action was detected
on diglycylglycine.

DISCUSSION

It is now possible to define the specificity requirements of leucine aminope-
tidase more precisely than heretofore. The data in Table I show that
the residues on the carboxyl end of the leucine group have some influence
on the rate of hydrolysis. The most rapid action is on the tripeptide (LGG)
and the dipeptide (LG). The markedly slower action on L-leucyl-L-glut-
amic acid as compared with LA indicates some inhibitory effect of the
second carboxyl group when it is near the sensitive bond. On the other
hand, the general configuration of the moiety attached to the carboxyl end
of the leucine residue cannot be highly critical, since it has been demon-
strated that L-leucyl-β-alanine is rapidly hydrolyzed by this enzyme (8).

The finding of a rapid action on GLA and GlLA was somewhat un-
expected. Nevertheless, in the presence of the free amino group, 1 residue
removed from the sensitive peptide bond does reduce the rate of hydrolysis
by about 40 per cent as compared with LA. The much slower action on
the tetrapeptide, GGLG, indicates that the sensitivity of the substrate is
greatly decreased as the distance between the sensitive peptide bond and
the free amino group is increased.
Leucine aminopeptidase has hitherto been regarded as the prototype of an aminopeptidase (9). This concept must now be revised to include our finding that the free amino group need not be present on the leucine residue which possesses the sensitive peptide bond. It has been recently suggested (10) that the role of heavy metals in peptidase action is the formation of a coordination compound linking the enzyme and the substrate. If this is so, one must assume that the ease with which the metal (Mn⁺⁺ or Mg⁺⁺) can form this bridge is a critical function of the distance from the sensitive bond.

**EXPERIMENTAL**

The enzyme experiments were performed as described in previous papers from this laboratory (4, 8) by means of the carboxyl titration method of Grassmann and Heyde (11). The leucine aminopeptidase was purified in the manner described by Smith and Bergmann (5). Some further purification was achieved by precipitation of the enzyme with 33 per cent acetone at room temperature, followed by dialysis and removal of the inactive precipitate. This procedure gave preparations essentially free of glycyl-L-leucine dipeptidase and tripeptidase activity as measured on diglycylglycine and prolidase.

L-Leucinamide Hydrochloride—A recrystallized preparation of L-leucine methyl ester hydrochloride (10 gm.) was allowed to stand in a pressure bottle at room temperature for 2 days with 50 cc. of anhydrous methanol which had previously been saturated with ammonia gas at 0°. The solution was then repeatedly concentrated in vacuo with methanol, and the crystals were filtered and washed with ether. Yield, 8.5 gm. After recrystallization from methanol-ether, thin plates were obtained; m.p. 236–237°.

C₆H₇ON₂Cl. Calculated. C 43.3, H 9.1, N 16.8
[α]D²⁰ = +9.5° (5% in water)

Behrens and Bergmann (12) found [α]D²⁰ = +9.25° for the corresponding acetate obtained by hydrogenation of carbobenzoxy-L-leucinamide.

Glycyl-L-leucinamide Hydrochloride

Carbobenzygycyl-L-leucine Methyl Ester—This compound has previously been described as an oil (13). The coupling was performed in the

² Although this compound has previously been described as the acetate obtained by hydrogenation of carbobenzyoxy-L-leucinamide (12), we are prompted to present the much simpler synthesis described above in view of the great utility of LA for studies of the enzymes of various tissues and sera. It should be noted that we have usually obtained somewhat faster enzymatic hydrolysis of the compound obtained by direct amidation than with the acetate prepared through the carbobenzyoxy intermediate.
manner given by Stahmann, Fruton, and Bergmann (13) with an ethereal solution of leucine methyl ester prepared in the usual way from 19.6 gm. of the hydrochloride and 26 gm. of carbobenzoxyglycyl chloride. After washing and drying the ethereal solution of the product, it was repeatedly concentrated in vacuo with dry ether. Yield, 24 gm. of needles on standing with petroleum ether. After recrystallization from ether-petroleum ether, the melting point was 64–66°.

\[ \text{C}_{17} \text{H}_{30} \text{O}_{3} \text{N}_2 \] (336.4). Calculated, N 8.3; found, N 8.3

**Carbobenzyglycyl-L-leucinamide**—The above ester (4 gm.) was amidated in methanol-ammonia in the usual manner. After repeated concentration with methanol, the compound crystallized on standing with a few drops of methanol. Yield, 3.6 gm. After recrystallization from methanol-ether and then from hot water, the melting point was 123–124°.

\[ \text{C}_{16} \text{H}_{31} \text{O}_{4} \text{N}_3 \] (321.3). Calculated, N, 13.1; found, N 13.0, 13.2

**Glycyl-L-leucinamide Hydrochloride**—The above amide (2.5 gm.) was hydrogenated in the usual manner in the presence of 10 cc. of \( \text{HCl} \), 35 cc. of methanol, and a palladium catalyst. After removal of the catalyst, the solution was concentrated in vacuo with ethanol and then with ether. Yield, 1.35 gm. After recrystallization from ethanol-ether, the melting point was 210° (slight browning).

\[ \text{C}_{16} \text{H}_{19} \text{O}_{2} \text{N}_3 \text{Cl} \] (223.7). Calculated, N 18.8; found N 18.8

\[ \alpha \] = -19.0° (95% in water)

**L-Glutamyl-L-leucinamide**

**Carbobenzyo-L-glutamyl-L-leucinamide**—To a dry ethyl acetate solution of L-leucine methyl ester prepared from 30 gm. of the hydrochloride, there were slowly added 35 gm. of carbobenzyo-L-glutamic acid anhydride (14). The slightly alkaline solution was allowed to stand at room temperature for 24 hours. It was then washed with dilute hydrochloric acid and with water, dried over \( \text{Na}_2\text{SO}_4 \), and concentrated to a thick oil in vacuo. 7 gm. of the oily ester were amidated in methanol-ammonia in the usual manner. After standing at room temperature for 3 days, the solution was concentrated in vacuo repeatedly with ether. The residue was extracted into hot ethyl acetate, filtered, and concentrated to dryness. Yield, 3.4 gm.; m.p. 165–169°, after recrystallization from hot water.

\[ \text{C}_{19} \text{H}_{30} \text{O}_{5} \text{N}_3 \] (393.4). Calculated, N 10.68; found, N 10.63

**L-Glutamyl-L-leucinamide—**1.0 gm. of the above compound was dissolved in methanol and hydrogenated in the presence of water and acetic acid.
The filtered solution was concentrated repeatedly with absolute ethanol. Yield, 0.5 gm.; m.p. 175-177°.

\[ \text{C}_{11}\text{H}_{21}\text{O}_{4}\text{N}_{3} \]

Calculated. C 51.0, H 8.2, N 16.2

259.3  


\[ \alpha_0^\circ = +6.7^\circ \text{ (2.1% in water)} \]

L-Leucyl-L-glutamic Acid—15 gm. of carbobenzyox-L-leucine hydrazide (15) were converted to the azide and coupled in ethyl acetate with glutamic acid diethyl ester prepared from 14 gm. of the hydrochloride. After standing at room temperature overnight, the ethyl acetate solution was worked up in the usual manner and concentrated in vacuo. The oily product was saponified at room temperature in 10 cc. of methanol and 140 cc. of M NaOH for 90 minutes. It was then acidified to Congo red and evaporated to dryness. The carbobenzyox-L-leucyl-L-glutamic acid was extracted into ethyl acetate, washed with water, extracted into M sodium bicarbonate, and acidified. The product was again dissolved in ethyl acetate, dried over Na\(_2\)SO\(_4\), and concentrated in vacuo. The substance was hydrogenated in methanol in the usual manner. Water was added during the hydrogenation to dissolve the crystalline peptide. After removal of the catalyst, the solution was concentrated to dryness with ethanol and then with ether. Yield, 1.1 gm.

\[ \text{C}_{11}\text{H}_{26}\text{O}_{4}\text{N}_{2} \]

Calculated. C 50.7, H 7.7, N 10.8

260.3  

Found. “ 50.8, “ 7.8, “ 10.8

\[ \alpha_0^\circ = +10.5^\circ \text{ (2% in M HCl)} \]

Fischer (16) found the same rotation with this compound prepared from optically active \(\alpha\)-bromoisocapronylglutamic acid.

Carbobenzyoxglycylglycyl-L-leucinamide—2.5 gm. of carbobenzyoxglycylglycyl-L-leucine methyl ester (17) were amidated in methanol ammonia in the usual way. After repeated concentration to dryness with methanol, the compound crystallized on gentle warming with water. Yield, 1.7 gm.; m.p. 181-182°.

\[ \text{C}_{18}\text{H}_{36}\text{O}_{4}\text{N}_{4} \] (378.4). Calculated, N 14.8; found, N 15.0

SUMMARY

1. Highly purified leucine aminopeptidase of hog intestinal mucosa hydrolyzes glycyl-L-leucinamide, L-glutamyl-L-leucinamide, glycylglycyl-DL-leucylglycine, and L-leucyl-L-glutamic acid in addition to the previously recognized substrates. The hydrolysis of all of these compounds takes place at the carboxyl end of the leucine residue. No endopeptidase action by this enzyme was detected.

2. The concept of aminopeptidase action is revised to include the fact...
that the free amino group need not be on the leucine residue which possesses the sensitive peptide bond.

3. The synthesis of a number of new derivatives and peptides of L-leucine is described.

BIBLIOGRAPHY

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