Substrate Specificity of the Gastrin-amidating Enzyme*

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As is the case with many other peptide hormones of the brain and gut, gastrin requires a carboxyl-terminal amide moiety for optimal biological activity. In the structure of progastrin, the carboxyl-terminal Phe of gastrin is followed by the sequence Gly83-Arg84-Arg85, which must be processed sequentially by an endoprotease, a carboxypeptidase, and an amidating enzyme to produce amidated bioactive gastrin. To examine the molecular determinants of peptide amidation in vivo, we mutated the wild-type Gly83 residue of progastrin to Ala83 and Ser83 and expressed the three progastrin DNAs in GH3 and MTC 6-23 endocrine cell lines. Although substantial quantities of amidated gastrin were seen in cells expressing wild-type progastrin, replacement of Gly83 with Ala83 completely abolished production of amidated gastrin when the cells were incubated in standard medium containing only L-alanine. In a similar fashion, cells expressing [Ser83]progastrin also demonstrated no production of amidated gastrin. When cells expressing [Ala83]- or [Ser83]progastrin were incubated in the presence of 1 mg/ml D-alanine or D-serine, respectively, a small but consistent amount of amidated gastrin production was detected (<1% of wild type). These data lead us to conclude that the amidating enzyme has a rigid substrate specificity for a glycine-extended precursor. Furthermore, this in vivo substrate specificity confirms the importance of the pro-S-α-hydrogen of the carboxyl-terminal glycine for enzyme-substrate recognition.

The structure of gastrin is similar to numerous other polypeptides of the brain and gut in that its carboxyl-terminal amino acid is amidated (1). As with most amidated peptides, the amide is an absolute requirement for biological activity (2). The post-translational processing of the carboxyterminus of progastrin begins with the endoproteolytic cleavage of Arg84-Arg85 by a prohormone convertase (Fig. 1A) (3, 4). The carboxyl-terminal arginine residues are subsequently removed via the action of carboxypeptidase H, forming a glycine-

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extended intermediate that serves as a substrate for peptidylglycine α-amidating monoxygenase (PAM).1 PAM is a bifunctional protein that contains a peptidylglycine α-hydroxylating monoxygenase domain and a peptidyl-α-hydroxylating glycine α-amidating lyase (5-9). The first step in the two-stage reaction is the hydroxylation of the α-carbon in glycine (Fig. 1B) via the action of peptidylglycine α-hydroxylating monoxygenase (10-13), followed by cleavage of the hydroxylated intermediate by peptidyl-α-hydroxylating glycine α-amidating lyase to form the peptide amide and glyoxylate (14). Existing data (15, 16) suggest that glycine-extended peptides or hormone-processing intermediates are the required substrates for the peptide amidation reaction, but this hypothesis has not been tested in vivo. Therefore, we undertook this study to determine the substrate specificity of amidation in vivo. Utilizing the retroviral expression vector pLJ, we expressed wild-type progastrin (Gly83) and two mutant progastrins (Ala83 and Ser83) in GH3 and MTC 6-23 cells and examined the effects of the mutations on the post-translational processing of progastrin.

MATERIALS AND METHODS

Gastrin DNA Constructs—Wild-type human gastrin cDNA (17) was a kind gift of Dr. E. Boel (University of Aarhus, Copenhagen). Gastrin DNA with site-specific mutations was constructed from this cDNA by the method of Kunkel (18). Trimmed human gastrin cDNA lacking its poly(A) signal was directionally ligated into M13mp18 by standard techniques. M13-gastrin viral phage were used to infect CJ 236 (Bio-Rad), an Escherichia coli dut-ung- strain that permits incorporation of uracil into newly synthesized DNA. Single-stranded uracil-containing template DNA was prepared from polyethylene glycol-precipitated phage by phenol/chloroform extraction. Oligonucleotides encoding the desired amino acid changes were synthesized on an Applied Biosystems DNA synthesizer, etamol-precipitated, and phosphorylated with T4 polynucleotide kinase. Aliquots (3 pmol) of the mutant oligonucleotide primer were annealed to 200 ng of uracil-containing M13-gastrin template. After annealing, T4 DNA polymerase and T4 DNA ligase were added in the presence of 0.4 mM dNTP to synthesize second-strand DNA lacking uracil bases. This duplex DNA was transformed into competent E. coli JM101, and phage DNA from individual plaques was prepared for subcloning into the expression vector pLJ. Each mutant was sequenced prior to subcloning by the method of Sanger et al. (19) to ensure correct synthesis of the desired mutant.

Infection of Endocrine Cell Lines—For the expression of wild-type and mutant gastrins, we utilized the pLJ retroviral vector and the psiCRE packaging cell system of Mulligan (20) as previously described (21). Briefly, wild-type and mutant gastrin DNAs prepared as described above were excised from the M13 vector with EcoRI and BamHI. A synthetic oligonucleotide adaptor was used to convert the 5'-EcoRI site to a SalI site to facilitate orientation-directed ligation of the DNA into pLJ. A triple ligation reaction was conducted for 6 h at 15 °C in a 10-μl volume containing 0.055 pmol of pLJ, 0.165 pmol of gastrin DNA, 0.48 pmol of the EcoRI-SalI adaptor, and 10 units of

1 The abbreviations used are: PAM, peptidylglycine α-amidating monoxygenase; PPLC, fast protein liquid chromatography.
T4 DNA ligase. Correct orientation of the gastrin-pLJ constructions was confirmed by restriction mapping. The gastrin-pLJ DNA was then transfected into the psiCRé packaging cell line by standard calcium phosphate coprecipitation (22), and transfected cells were selected by maintaining the culture in medium containing the neomycin analog G418 (1 mg/ml). Medium from the selected cell lines containing gastrin-pLJ DNA was collected and stored at -70 °C for later use as viral stock for infection of endocrine cell lines.

The two endocrine cell lines used for our expression studies were selected on the basis of some of their known properties. GH3 cells are from a rat pituitary tumor and express growth hormone and prolactin (23). Previous studies have indicated that GH3 cells process progastrin (24), pro-pancreatic polypeptide (25), and proinsulinoma B-cell line (26), thus demonstrating that they have the enzymatic activities necessary for dibasic cleavages and carboxyl-terminal amidation. MTC 6-23 cells are derived from a rat medullary thyroid carcinoma (27) and have been shown to express similar enzymes that process progastrin (21) as well as endogenous calcitonin and neurotensin. MTC 6-23 cells were grown in Dulbecco's modified Eagle's medium containing 15% horse serum, whereas GH3 cells were grown in the same medium with 10% horse serum and 5% fetal calf serum. For infection, gastrin-pLJ viral stock prepared as noted above was filtered with a 0.22-μm filter and added to 30-50% confluent target endocrine cells in the presence of 8 μg/ml Polybrene (Aldrich) for 4 h. The infected cells were grown in complete medium at 37 °C for 48 h and then selected in G418 (1 mg/ml) containing G418 modified Eagle's medium containing 1 mg/ml D-alanine (Sigma) for 48-72 h prior to ligation or in serine-free Select-amine (GIBCO) containing 1 mg/ml D-serine (Sigma). Populations of cells (≥200 clones/mutant) rather than individual clones were chosen for study to normalize the expression of the wild-type and mutant gastrin DNAs and thus to eliminate the differences that could be observed in the post-translational processing of progastrins in single clones with varying levels of gastrin DNA expression.

To confirm that the infected endocrine cell lines contained the exact mutant that we had transfected into the psiCRé packaging cells, we obtained the nucleotide sequence of the gastrin cDNA integrated into the target cell genome. DNA was prepared by standard techniques (28) from confluent MTC 6-23 and GH3 cells expressing specific mutant gastrins. Using oligonucleotides complementary to each end of the inserted human gastrin cDNA, we amplified the entire coding region of the integrated mutant gastrin by polymerase chain reaction (29). The appropriate 400-base pair band was obtained on gel electrophoresis. The oligonucleotide primers contained internal EcoRI and BamHI restriction sites that were used to cut the polyoma virus DNA to subclone it into M13 for sequencing by the method of Sanger et al. (19). We confirmed that in each case the desired mutation was present and that no other genetic alterations had occurred.

**RNA Analysis**—to confirm the expression of the various mutant DNAs, we examined the RNAs produced in the various transfected cell lines via Northern hybridization. Total RNA was prepared from each cell line by the acid/phenol method of Chomczynski and Sacchi (30). Total RNA (10 μg) was electrophoresed on 1% formaldehyde-agarose gels and transferred to Hybond-N filters (Amersham Corp.) using a Posiblot apparatus (Stratagene). RNA was UV-cross-linked to filters and prehybridized in 5 × sodium chloride/sodium citrate (SSC), 5 × Denhardt's solution, 0.5% SDS, and 100 μg/ml salmon sperm DNA for 2 h at 65 °C. Radiolabeled random-primed cDNA probes were prepared from human gastrin and β-actin cDNAs with a specific activity of 1-3 × 10⁸ cpmpμg (18). Probes were added to the prehybridization mixture and hybridized for 16 h at 65 °C. Filters were washed, with the most stringent wash being 0.1 × SSC, 1% SDS at 65 °C for 1 h. After air drying, the filters were exposed to radiographic film at -70 °C.

**Analysis of Gastrin Products**—Cells were trypsin-treated, dispersed by pipetting, and counted in a Coulter counter. Gastrin-expressing cells were then extracted in boiling water, centrifuged, and stored at -20 °C prior to radioimmunoassay. Extracts were assayed for carboxyl-terminally amidated gastrins with antiserum 5135, which recognizes both sulfated and unsulfated forms of amidated gastrin. We confirmed that in each case the desired mutation was present and that no other genetic alterations had occurred.

The molecular forms of gastrin produced in each cell line were determined by fast protein liquid chromatography (FPLC) using an anion exchange column (21). Mono-Q anion-exchange FPLC columns (Pharmacia LKB Biotechnology Inc.) were equilibrated with Buffer A (50 mm Tris, pH 8.2, 10% acetonitrile), and samples were eluted with a gradient of the same buffer containing 1 m NaCl. Calibration of both columns was preformed as previously described for both un sulfated and Tyrα-sulfated gastrins (21, 31).

**RESULTS**

We constructed two mutant progastrins with the wild-type Glyα residue changed to Alaα or Serα. The nucleotide sequence of each mutant DNA was confirmed prior to ligation into the pLJ expression vector. Total RNA was prepared from GH3 and MTC 6-23 cells that had been infected with the pLJ vector containing either wild-type or mutant gastrin DNA inserts. As shown in Fig. 2, comparably high levels of gastrin-pLJ RNA were detected in each mutant cell line as judged by Northern blot analysis. The molecular size of the gastrin-pLJ

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**Fig. 1. Progastrin-processing and antibody recognition sites.** In A, the progastrin molecule is shown with the amino- and carboxyl-terminal extensions and dibasic cleavage sites Argα-Argα, Lysα-Lysα, and Argα-Argα. Gastrin heptadecapeptide (G-17) and gastrin tetraacapeptide (G-34) are overlined with solid bars. Carboxyl-terminal processing is initiated by a prohormone convertase that removes the amino- and carboxyl-terminal extensions. The remaining basic amino acid residues are removed by carboxypeptidase H, and the resultant glycine-extended intermediate serves as a substrate for PAM, which results in the formation of amidated gastrin. Sulfation of Tyrα generates sulfated forms of amidated gastrin. Endoproteolytic cleavage at Lysα-Lysα results in the formation of G-17. Sulfated and unsulfated amidated gastrins were measured with antibody 5135, which cross-reacts <1% with glycine-extended gastrins, by radioimmunoassay. In B, the two-step reaction necessary for peptide amidation is depicted showing the selective attack of peptidylglycine α-hydroxylating monoxygenase (PHM) on the pro-S-hydrogen of glycine to generate a hydroxylated intermediate, which is then converted into amidated gastrin and glyoxylate via the action of peptidyl-α-hydroxycarboxyl peptide α-amidating lyase (PAM). The nitrogen atom labeled with an asterisk in the peptide amide is derived from the glycine moiety in the precursor molecule.
panels) by either cell line. We confirmed that the specific mutant wild-type progastrin was expressed in wild-type and mutant progastrins. Northern hybridization was performed with random primed \textsuperscript{32}P-labeled gastrin and \textbeta-actin cDNAs (specific activity of 1–3 \times 10^{6} cpn/\mu g). GH3 (upper panel) and MTC 6-23 (lower panel) cells were analyzed, and the conditions for the hybridization and subsequent washes are described under "Materials and Methods." Filters hybridized with gastrin cDNA probes (left panels) were exposed to x-ray film for 90 h, whereas the exposure time for the same filters after stripping and hybridization with \textbeta-actin probes (right panels) was 18 h. RNAs from GH3 cells expressing [Ser\textsuperscript{R}]progastrin (lane A) and [Ala\textsuperscript{R}]progastrin (lane B) as well as wild-type progastrin (lane D) are depicted. RNAs from control GH cells are depicted in lane C. For MTC 6-23 cells, wild-type and [Ser\textsuperscript{R}]- and [Ala\textsuperscript{R}]progastrins are depicted in lanes A–C, respectively. Control MTC 6-23 cells do not express progastrin as shown previously (21).

**TABLE I**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Standard medium</th>
<th>D-Ala medium</th>
<th>D-Ser medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH3</td>
<td>115 ± 16</td>
<td>50 ± 8.3</td>
<td>476 ± 51</td>
</tr>
<tr>
<td>Ala\textsuperscript{R}</td>
<td>0</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Ser\textsuperscript{R}</td>
<td>0</td>
<td>0.8 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>MTC 6-23</td>
<td>145 ± 18</td>
<td>520 ± 30</td>
<td>920 ± 100</td>
</tr>
<tr>
<td>Wild-type Gly\textsuperscript{R}</td>
<td>0</td>
<td>0.5 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

Amidated gastrin production in cell extracts of various transformed cell lines

Amidated gastrin was assayed with antiserum 5135. The data were obtained from \( n \geq 6 \) samples. Values are means ± S.E.

transcripts corresponded to the predicted length of 5.2 kilobases, and endogenous rat gastrin DNA was not transcribed by either cell line. We confirmed that the specific mutant gastrin DNA had not undergone any changes during the retroviral integration process by sequencing each mutant preprogastrin DNA in its entirety for all infected GH3 and MTC 6-23 cells.

As shown in Table I, GH3 and MTC 6-23 cells transfected with the wild-type progastrin DNA produced substantial amounts of fully processed amidated gastrin. Mutation of Gly\textsuperscript{R} to either Ala\textsuperscript{R} or Ser\textsuperscript{R} completely abolished production of amidated gastrin immunoreactivity. When MTC 6-23 and GH3 cells containing [Ala\textsuperscript{R}]progastrin were grown in medium containing D-alanine, small but consistently measurable quantities of amidated gastrin immunoreactivity were observed. In a similar fashion, GH3 and MTC 6-23 cells infected with [Ser\textsuperscript{R}]progastrin and grown in medium with D-serine were noted to contain amidated gastrin immunoreactivity. For further characterization, the amidated gastrin immunoreactivity in cell extracts was analyzed by anion-exchange FPLC. As depicted in Fig. 3, the major peaks of immunoreactivity coeluted with known molecular forms of amidated gastrin. Furthermore, the distribution of molecular forms was similar for wild-type and [Ala\textsuperscript{R}]- and [Ser\textsuperscript{R}]progastrins. As in the case of the GH3 cells, the immunoreactivity in MTC 6-23 cell extracts also coeluted with the known molecular forms of amidated gastrin. The distribution of amidated molecular forms appeared to be similar for wild-type and [Ala\textsuperscript{R}]- and [Ser\textsuperscript{R}]progastrins, except that there was less gastrin tetradecapeptide (G-14) seen in [Ala\textsuperscript{R}]- and [Ser\textsuperscript{R}]progastrins than in wild-type progastrin (Fig. 4).

We also sought to determine the effects of the D-alanine- and D-serine-containing media on production and processing of amidated gastrin in cells expressing wild-type progastrin. The production of amidated gastrin was slightly decreased in GH3 cells grown in D-alanine-containing medium, but increased in D-serine-containing medium, whereas both D-serine- and D-alanine-containing media increased the production of amidated gastrin in MTC 6-23 cell extracts (Table I). When this immunoreactivity was analyzed by FPLC, there were no significant differences in the distribution of amidated gastrin molecular forms in GH3 (Fig. 5) and MTC 6-23 (Fig. 6) cell extracts.
FIG. 4. Characterization of gastrin molecular forms in MTC 6-23 cell extracts expressing wild-type and mutant progastrins by anion-exchange chromatography. Cells expressing [Ala]$^9$- and [Ser]$^9$-progastrins were grown in D-Ala- or D-Ser-containing medium, respectively. Cell extracts were applied to an HR 5/5 Mono-Q FPLC anion-exchange column as described for Fig. 3. The chromatograms shown are representative of at least three for each cell line. G34S, sulfated G-34; G17S, sulfated G-17.

FIG. 5. Characterization of gastrin molecular forms in GH3 cell extracts expressing wild-type gastrin incubated in either D-alanine-containing (upper) or D-serine-containing (lower) medium by anion-exchange chromatography. Cell extracts were treated as described for Fig. 3. The chromatograms shown are representative of at least three for each cell line. G34S, sulfated G-34; G17S, sulfated G-17.

FIG. 6. Characterization of amidated gastrin immunoreactivity in MTC 6-23 cell extracts expressing wild-type gastrin incubated in either D-alanine-containing (upper) or D-serine-containing (lower) medium. Cell extracts were applied to an HR 5/5 Mono-Q FPLC anion-exchange column as described for Fig. 3. The chromatograms shown are representative of at least three for each cell line. G34S, sulfated G-34; G17S, sulfated G-17.

DISCUSSION

The structure of gastrin is similar to numerous other polypeptides of the brain and gut in that its carboxyl-terminal amino acid is amidated (1). As with most amidated peptides, the amide is a requirement for optimal biological activity (22). The carboxyl-terminal amino acid of gastrin is extended by glycine in the structure of progastrin, and glycine-extended progastrin-processing intermediates serve as the substrates for PAM, resulting in the production of amidated gastrin (32). PAM is a bifunctional protein that contains peptidylglycine $\alpha$-hydroxylating monoxygenase or monoxygenase activity that catalyzes the formation of a hydroxyl intermediate. This intermediate serves as a substrate for peptidyl-$\alpha$-hydroxylglycine $\alpha$-amidating lyase, which catalyzes the formation of the peptide amide and glyoxylate (5–14). It is felt that PAM has a rigid substrate specificity for glycine-extended peptides (15, 16), but this has not been clearly demonstrated in vivo. For instance, the precursor for the amidated peptide galanin contains a glycine residue that follows the penultimate COOH-terminal amino acid in the rat and pig, but human progalanin contains a serine residue (33–36). We therefore hypothesized that serine- and alanine-extended processing intermediates could serve as substrates for PAM and allow for the production of amidated peptides.

For our studies, we utilized site-directed mutagenesis to change Gly$^9$ in progastrin to either Ala$^9$ or Ser$^9$. The wild-type and mutant progastrins were successfully expressed in GH3 and MTC 6-23 cells as judged by mRNA expression and neomycin resistance of the cells. Although substantial quantities of amidated gastrin were seen in cells expressing wild-type progastrin, no amidated gastrin production was seen in either mutant progastrin grown in standard medium containing L-amino acids. These findings are in agreement with in vitro data demonstrating that a tripeptide utilizing L-alanine as a substrate could not be amidated (15). This is also consistent with the recent report demonstrating that serine-containing human progalanin is not amidated (37, 38) and confirms that PAM has a rigid substrate specificity for glycine-extended precursors.
Fig. 7. Three-dimensional representation of pro-S-hydrogen (H₃) and pro-R-hydrogen (Hₛ) of glycine and l- and d-isomers of alanine and serine. The spacial relationship of the pro-S-hydrogen of glycine to the carboxyl and amino groups attached to the a-carbon is the same as the relationship of the methyl group of L-alanine and the methanol group of L-serine to their carboxyl and amino groups. However, when in the configuration, the a-hydrogen of D-alanine and D-serine are in a spacial relationship similar to that of the pro-S-hydrogen of glycine.

Since previous studies have demonstrated that PAM is capable of using tripeptides such as D-Tyr-Val-D-Ala as substrates in vitro (16), we sought to determine if a D-amino acid substrate could serve as a PAM substrate in vivo. Utilizing medium with 1 mg/ml D-alanine, we were able to detect a small amount (<1% of wild type) of amidated gastrin immunoreactivity in both GH3 and MTC 6-23 cells expressing [Ala₃]progastrin. Although D-Tyr-Val-D-Ser has previously been shown not to be a substrate for PAM in vitro (16), we were able to detect amidated gastrin immunoreactivity in cells expressing [Ser₃]progastrin grown in 1 mg/ml D-serine. This indicates that d-serine- and d-alanine-containing peptides can serve as peptide substrates for PAM in vivo. Confirmation that the observed immunoreactivity was amidated gastrin was provided by FPLC analysis of [Ala₃]- and [Ser₃]progastrin-producing cells. The observation that d-alanine and d-serine (but not L-alanine and L-serine) can serve as PAM substrates suggests that the orientation of the a-carbon–hydrogen bond is important for enzyme-substrate recognition (Fig. 7). Because the side chain attached to the a-carbon of glycine is a single hydrogen atom, glycine does not exist as a stereoisomer. However, it is possible to label selectively a single a-carbon–hydrogen, resulting in the formation of pro-S and pro-R forms of glycine. Using these substrates, peptide-glycyl a-hydroxylating monooxygenase selectively utilizes the pro-S-hydrogen (but not the pro-R-hydrogen) in glycine (39-41) to form a hydroxyl intermediate. Additionally, it appears that peptide-d-α-hydroxylglycine a-amidating lyase, like peptide-glycyl a-hydroxylating monooxygenase, will only catalyze the formation of peptide amides from hydroxyl intermediates in the pro-S (but not pro-R) configuration (41). Our in vitro data are consistent with the notion that peptide-glycyl a-hydroxylating monooxygenase and peptide-d-α-hydroxylglycine a-amidating lyase selectively attack the a-hydrogen of carboxyl-terminal amino acids in the pro-S configuration since we could detect amidated gastrin production with D-amino acids (but not L-amino acids) that place the a-hydrogen in the pro-S configuration (Fig. 7).

Although our results suggest that D-amino acids can be incorporated into peptides produced in these tumor cell lines, it should be noted that the total production of amidated gastrin with [Ala₃]- and [Ser₃]progastrins in the presence of D-amino acids was much less (<1%) than that observed with wild-type gastrin. One possible explanation for this observation is the highly stereoselective nature of the steps required for the incorporation of amino acids into proteins. These processes demonstrate preferences for L-amino acids in transport across the cell membrane, in aminoacylation of the tRNA, in elongation of proteins during synthesis, and in binding of the amino acid-tRNA complex to the ribosome (42, 43). It has been calculated that these cumulative preferences yield a discrimination for the L-isomer of ~10⁴. Thus, the amount of D-amino acid incorporation would be expected to be low. Nevertheless, small amounts of D-amino acids have been detected in a variety of mammalian proteins and are part of the primary structure of the neuropeptide dermorphin (44, 45), although the mechanism of its D-isomer incorporation remains undefined (46). It is interesting to note that despite the expectation that D-amino acid substitution might interfere with production of cellular proteins in a generalized fashion, we did not see a marked decrease in amidated gastrin production in cells expressing wild-type progastrin incubated in medium containing D-serine or D-alanine. Another factor that may contribute to the relatively diminished expression of amidated gastrin in [Ala₃]- or [Ser₃]progastrin-expressing cells is that although we utilized medium without L-alanine or L-serine in our studies to enhance the incorporation of the D-isomers, both of these L-isomers can be synthesized from the glycolytic intermediates pyruvate (alanine) and 3-phosphoglycerate (serine). These endogenous L-amino acids could then compete with the exogenous D-isomers for incorporation into the mutant progastrins and ultimately diminish amidated gastrin production. Furthermore, D-amino acid-containing peptides such as D-Tyr-Val-D-Ala are poor PAM substrates in vitro (16); therefore, the in vitro conversion of a D-amino acid-containing progastrin substrate to amidated gastrin would be inefficient at best. Our results, combined with data using nonpeptide substrates (15, 16), confirm that the spacial relationship of the a-carbon, hydrogen, and nitrogen atoms of the glycine moiety is important for enzyme-substrate recognition in vitro and in vivo.

REFERENCES
Gastrin-amidating Enzyme


