*Proc. Natl. Acad. Sci. USA*
Vol. 74, No. 3, pp. 975–978, March 1977
Biochemistry

# Translation of melittin messenger RNA *in vitro* yields a product terminating with glutaminylglycine rather than with glutaminamide

**(precursor/wheat germ extract/peptide biosynthesis)**

GERDA SUCHANEK AND GÜNTHER KREIL

Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, 5020 Salzburg, Austria

*Communicated by Donald F. Steiner, December 30, 1976*

ABSTRACT **Melittin messenger RNA from queen bee venom glands has been translated in a cell-free system from wheat germ. A product larger than promelittin is formed which has the carboxy-terminal sequence -Gln-Gln-GlyCOOH. Melittin and promelittin from venom glands terminate in -Gln-GlnCONH$_2$. The possible role of the extra glycine residue in the formation of a COOH-terminal amide via a transamidase-like reaction is discussed.**

Tuppy and Michl demonstrated some 20 years ago that the peptide hormone oxytocin does not contain a free $\alpha$-carboxyl group but instead terminates with an amino acid amide (1). Many peptides have since been described that also have a COOH-terminus blocked in this way. So far, at least 10 different amino acids have been found to occur at such amidated termini. This modification of the $\alpha$-carboxyl group has hitherto only been detected in peptides containing fewer than 40 residues, but it is by no means universal for these small molecules. On the contrary, sometimes even closely related peptides may or may not be amidated. For example, in the case of the two hormones secretin and glucagon, which exhibit similar chain length and considerable sequence homology, only the former has a blocked carboxyl end (2, 3).

The mechanism of this amidation, which is undoubtedly a post-translational event, is at present unknown. From the variety of COOH-terminal residues that may in one case be amidated, in others not, it appears unlikely that a free $\alpha$-carboxyl group and the side chain of the terminal amino acid could be a specific recognition site for this type of modification.

During the past years we have studied the biosynthesis of melittin, the main component of honeybee venom. This peptide terminates in glutaminamide (4). A precursor of melittin, termed promelittin, has been detected in venom glands (5, 6) and has the same COOH-terminal end. The messenger RNA for melittin has recently been isolated from the venom glands of young queen bees (7). Injection of this RNA into oocytes of *Xenopus laevis* led to the formation of a polypeptide closely resembling promelittin, but it appeared to lack the COOH-terminal amide (8). More recently, melittin messenger RNA has been translated in a cell-free system prepared from mammalian cells (9), and evidence has been presented that its *in vitro* product is considerably larger than promelittin. The bulk of the extra residues are linked to the amino-terminus of promelittin. By analogy with similar precursors for other secretory polypeptides (10–16), this product has been designated prepromelittin. As compared to venom gland products, it is not amidated at the carboxyl end but instead contains one to a few extra residues (9).

We now report the COOH-terminal structure of prepromelittin obtained by translation of melittin messenger RNA in a cell-free system from wheat germ. The terminal sequence is shown to be -Gln-Gln-GlyCOOH, which contrasts with that of promelittin and melittin from venom glands, which terminate in -Gln-GlnCONH$_2$.

## MATERIALS AND METHODS

Tritium-labeled amino acids of the highest specific activity currently available were obtained from The Radiochemical Centre (Amersham, England). Reference peptides were purchased from Bachem AG (Liestal, Switzerland). The proteolytic enzymes were commercial products (Sigma, St. Louis), with the exception of staphylococcal protease, which was a gift of Dr. G. R. Drapeau (University of Montreal, Canada).

**Preparation of Venom Gland RNA.** Total RNA was isolated from venom glands of newly emerged queen bees as described (7, 8). For most experiments this RNA was used without further fractionation. In some instances, however, the cell-free system was programmed with the poly(A)-containing RNAs obtained by chromatography on poly(dT)-cellulose. It has been shown that in the latter preparations melittin messenger RNA is the predominant species (7).

**Protein Synthesis Assay.** The assays were carried out in a final volume of 50 $\mu$l. Incubation mixtures were 28 mM in Hepes (*N*-2-hydroxypiperazine-*N'*-2-ethanesulfonic acid) (pH 7.4), 2 mM in dithiothreitol, 1 mM in ATP, 25 $\mu$M in GTP, 8 mM in creatine phosphate, 88 mM in KCl, 3 mM in magnesium acetate, and 50 $\mu$M in each of 19 unlabeled amino acids. The incubation mixtures also contained 20 $\mu$l of wheat germ extract, 2 $\mu$g of creatine kinase (Boehringer, Mannheim), 20 $\mu$g of total venom gland RNA or 0.2 $\mu$g of poly(A)-containing RNA, and 10–50 $\mu$Ci of one tritiated amino acid. Samples were incubated at 28° for 60 min. An aliquot of 5 $\mu$l was then removed from each sample and analyzed for radioactivity insoluble in hot trichloroacetic acid (17).

**Product Analysis.** Reaction mixtures were diluted to 300 $\mu$l with water, adjusted to pH 8.5 with dilute ammonia, and then extracted three times with an equal volume of *n*-butanol. The interface material was collected, washed several times with water containing unlabeled amino acids, and subsequently dried. This fraction, which contained, besides denatured wheat germ proteins, all of the radioactive prepromelittin, was used for the enzymatic digestions without further purification. All the analytical procedures, including enzymatic hydrolysis, fractionation by high voltage paper electrophoresis at different pH values, stepwise Edman degradation, etc., have been described in previous publications (6, 8, 9). Further details are given in the legends to the figures.

## RESULTS

Peptic digests of melittin or promelittin contain a basic heptapeptide which is derived from the carboxyl end. The structure of this fragment is: Ile-Lys-Arg-Lys-Arg-Gln-GlnCONH$_2$ (4,

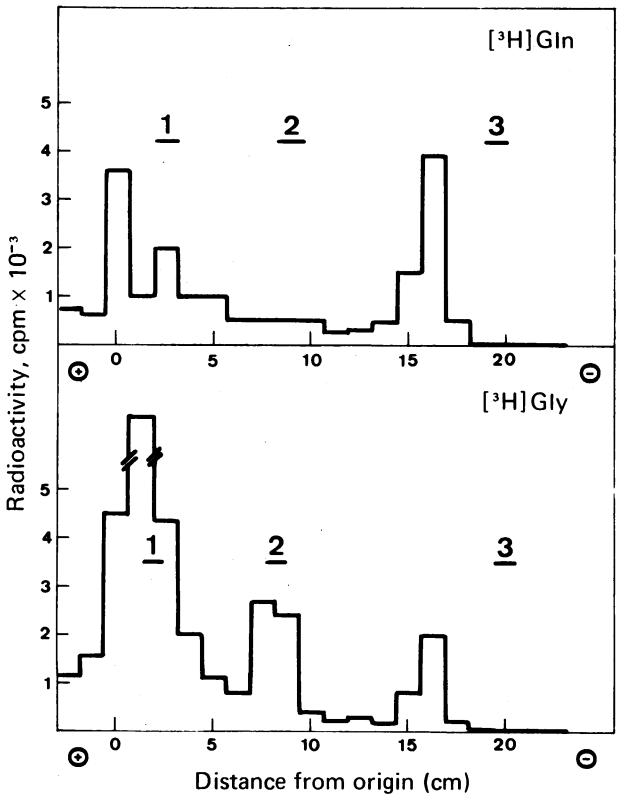


FIG. 1. Analysis of the cell-free products labeled either with [$^3$H]glutamine or [$^3$H]glycine. *Upper.* Each assay mixture contained 30 $\mu$Ci of [$^3$H]glutamine (26 Ci/mmol). Interface material was isolated from three such assays, as described in *Materials and Methods*, and digested with 0.1 mg of pepsin in 0.5 ml of dilute HCl (pH 1.6, 37°, 4 hr). The digest was dried and fractionated by high voltage paper electrophoresis at pH 4.8 (1% pyridine/acetate buffer, Whatman 3MM paper). The electrophoretogram was cut into 1.2-cm sections, and radioactivity of each was determined in a liquid scintillation counter (toluene-based scintillator). *Lower.* Each assay mixture contained 50 $\mu$Ci of [$^3$H]glycine (5.3 Ci/mmol). From two such assays, interface material was collected and processed as described for the sample labeled with [$^3$H]glutamine. Reference substances: (*1*) leucine, (2) a peptic melittin fragment containing residues 7–13, (*3*) lysine.

6). Upon paper electrophoresis at pH 4.8, this fragment, with a net charge of +5, migrates ahead of free lysine (mobility relative to lysine, $R_{Lys}$ = 1.1–1.2). In the peptic hydrolysates of prepromelittin obtained from the wheat germ system, this fragment could not be detected. Instead, a peptide with lower mobility ($R_{Lys}$ = about 0.80) was present on the electrophoretogram, which was presumed to be larger and/or less positively charged than the COOH-terminal fragment of melittin. The electrophoretic pattern observed with a peptic digest of prepromelittin labeled with [$^3$H]glutamine is shown in the upper panel of Fig. 1. The basic fragment located at about 16 cm from the origin was eluted and sequentially degraded by the Edman procedure. In each of the first five steps, less than 1% of the total radioactivity could be extracted into butylacetate. The fragment was still basic after four rounds of Edman degradation ($R_{Lys}$ = 0.46) and became neutral after the fifth one. At step 6, 21% of the total label (1250 cpm) was extractable with butylacetate, which demonstrated the presence of one glutamine residue in this position. The material remaining in the aqueous layer was then fractionated by paper electrophoresis at pH 1.6, using glutaminylglycine and glutaminylglutamine as reference peptides. Of the total label present on the electrophoretogram, about half comigrated with synthetic glutaminylglycine, while the remainder stayed at the origin. This latter material clearly lacked any free amino group. The compound migrating like glutaminylglycine was eluted and further degraded. At this seventh Edman step, 71% of the remaining radioactivity (980 cpm) could be extracted into the organic phase.

This experiment demonstrated that the basic fragment shown in Fig. 1 contains glutamine in positions 6 and 7, and furthermore indicated that residue 8 is glycine. The low yields obtained from the Edman degradation are probably due to the fact that the fragments with $NH_2$-terminal glutamine, present after the fifth and sixth step, have a marked tendency to cyclize to the corresponding pyrrolidone-carboxylic acid derivatives.

In two experiments of the same type as above, prepromelittin labeled either with [$^3$H]isoleucine or [$^3$H]lysine was analyzed. Again, peptic hydrolysates were prepared and fractionated by paper electrophoresis at pH 4.8 to obtain the basic fragment of $R_{Lys}$ at about 0.80. By stepwise Edman degradation it could be shown that this fragment contained amino-terminal isoleucine and that lysine was present in positions 2 and 4. The homology between the sequence of this fragment of prepromelittin and the COOH-terminal region of melittin was thus established.

The experiments with labeled glutamine had indicated that the basic fragment isolated from peptic digests of prepromelittin terminates in glycine. This notion was corroborated by analyzing prepromelittin containing radioactive glycine. Peptic hydrolysis and subsequent fractionation by paper electrophoresis yielded the pattern shown in the lower panel of Fig. 1. Two basic peptides containing radioactive glycine were present in this case: a more slowly moving peptide ($R_{Lys}$ = 0.36), which corresponds to part of the melittin sequence (residues 7 through 13 of melittin with glycine in position 12), and the basic fragment of $R_{Lys}$ = 0.80, which was also observed in the other experiments. The latter peptide was eluted and in this instance further digested with trypsin. The resulting neutral peptide was subjected to one cycle of Edman degradation and then fractionated by paper electrophoresis at pH 1.6. As shown in Fig. 2 (*upper*), the bulk of the radioactivity comigrated with authentic glutaminylglycine. This material was eluted, again treated by the Edman procedure, and analyzed by paper electrophoresis under the same conditions (Fig. 2 *lower*). After this second Edman step virtually all of the label comigrated with free glycine.

These results showed that the basic fragment present in peptic hydrolysates of prepromelittin had the partial structure: $NH_2$-Ile-Lys-X-Lys-X-Gln-Gln-Gly-COOH. It appears likely that this sequence also corresponds to the carboxyl end of prepromelittin. However, because of the broad specificity of pepsin, the COOH-terminal glycine of the basic fragment could have been generated during the enzymatic hydrolysis and the presence of additional residues at the carboxyl end of prepromelittin can therefore not be excluded. A direct determination of the terminal residue of prepromelittin by the hydrazinolysis procedure (18) is not feasible because our samples always contain traces of free amino acids, i.e., some free, radioactive glycine in the glycine incorporation experiments. We have therefore chosen an indirect route to check whether the above fragment indeed represents the carboxyl end of prepromelittin.

Several years ago, a protease was isolated from culture filtrates of *Staphylococcus aureus* that cleaves specifically after acidic residues (19). Incubation of promelittin with this enzyme yields melittin with an extra alanine residue at the amino end (unpublished experiments). Using the same conditions for enzymatic hydrolysis, a melittin-like fragment has been obtained

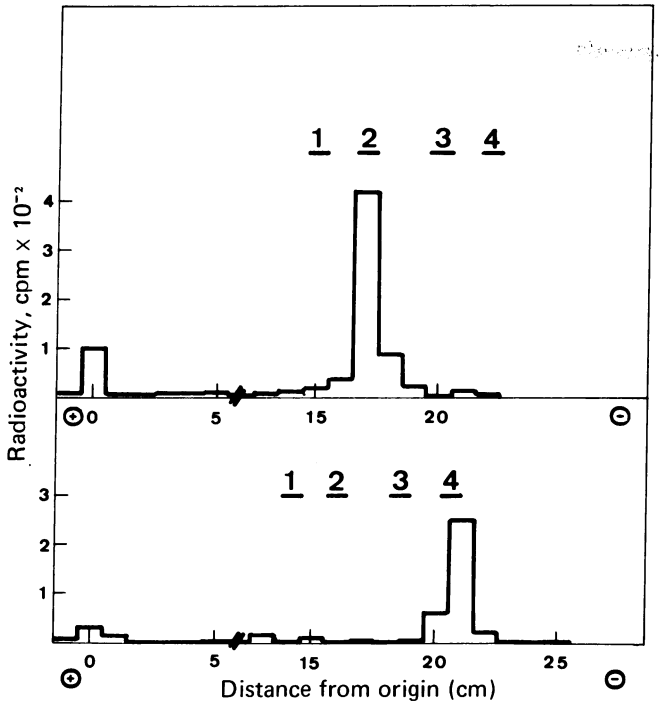


FIG. 2. Analysis of the basic fragment labeled with [$^3$H]glycine. From the experiment shown in Fig. 1, *lower panel*, the fragment migrating about 16 cm from the origin was eluted and further digested with trypsin (50 $\mu$g of trypsin in 0.2 ml of dilute ammonia, pH 8.0, 37°, 2.5 hr). The digest was again separated by electrophoresis at pH 4.8, and the now neutral peptide was subjected to Edman degradation. *Upper*. Electrophoretogram of the water phase after the first Edman step. The material comigrating with glutaminylglycine was eluted and subjected to a second Edman cycle. The resulting water phase was again fractionated by paper electrophoresis (*lower*). Electrophoresis was performed at pH 1.6 (7.5% acetic acid adjusted with formic acid). Electrophoretograms were cut into sections 1 cm wide, and radioactivity of each was determined in a liquid scintillation counter. Reference substances: (*1*) glutaminylglutamine, (2) glutaminylglycine, (*3*) glycylglycine, (*4*) glycine.

from prepromelittin synthesized in the cell-free system from mammalian cells (9). It has been shown that this fragment can be extracted into *n*-butanol and also behaves like melittin upon paper electrophoresis and chromatography.

To obtain information about the carboxyl end, we again prepared prepromelittin in the wheat germ system in the presence of labeled glycine. It was then treated with staphylococcal protease, and the melittin-like fragment was isolated by butanol extraction and paper electrophoresis at pH 3.5 as described (9). Subsequently, this fragment was further digested with trypsin and the resulting hydrolysate was fractionated by paper electrophoresis at pH 4.8. As in the previous experiment, a neutral peptide containing radioactive glycine was obtained that was indistinguishable from glutaminylglycine after one Edman step, and that yielded free glycine after the second step. The same tripeptide with COOH-terminal glycine can thus be isolated not only from peptic plus tryptic hydrolysates of prepromelittin, but also after the sequential action of staphylococcal protease and trypsin. In the latter case, in particular, neither of these two enzymes can be expected to cleave after a glycine residue; consequently, this amino acid must be the carboxy-terminal residue of prepromelittin.

## DISCUSSION

The experiments reported in this paper demonstrate that prepromelittin, synthesized in a cell-free system from wheat germ programmed with melittin messenger RNA, terminates in the sequence -Gln-Gln-GlyCOOH. Our earlier results on the translation of this messenger RNA in frog oocytes (8) and in a cell-free system prepared from mammalian cells (9) suggest that in these instances products with the same COOH-terminal sequence were formed. For example, in both these systems the basic fragment shown in Fig. 1 was observed in peptic hydrolysates. Experiments with the mammalian system had in fact already demonstrated that a short extension of one to a few residues is present at the carboxyl end of prepromelittin. These observations plus the fact that the wheat germ system has been shown to translate a number of animal messenger RNAs correctly make it unlikely that the observed COOH-terminal structure represents an artifact or is due to premature chain termination. In particular, synthesis of incomplete chains is not likely to occur with a messenger RNA containing only about 450 nucleotides (7), of which less than 250 are translated to yield prepromelittin. We therefore assume that the formation of the terminal amide bond of melittin in honeybee venom glands also proceeds via a precursor that contains an extra glycine residue at the carboxyl end. Whether a precursor with this primary COOH-terminal structure can be detected in venom glands remains to be investigated.

It is our present conjecture that a COOH-terminal glycine represents the recognition site for a venom gland enzyme that exchanges glycine by ammonia. Several mechanisms for such a reaction could be envisaged, of which transamidation with, for example, glutamine appears to be the most likely possibility. The venom gland also synthesizes peptides, albeit in small amounts compared to melittin, that terminate with asparaginamide and histidinamide, respectively (20–22). The proposed mechanism would of course be independent of the nature of the COOH-terminal residue in the final product and would only require the presence of a terminal glycine in the respective precursor polypeptide. In this context one might ask whether the published sequence data of peptides and their precursors provide any indication that a mechanism similar to that which we propose for melittin may operate in other cases as well. An example can indeed be quoted where an analogous situation may exist: the pituitary peptide $\alpha$-melanotropin is considered to be derived from corticotropin (23), of which it represents the first 13 residues. If the release of $\alpha$-melanotropin from its precursor proceeds via the action of trypsin- and carboxypeptidase B-like activities, as has been found for proinsulin and other prohormones (24), an $\alpha$-melanotropin precursor would first be generated with the COOH-terminal structure -Val-GlyCOOH. An enzyme similar to the one postulated to act in honeybee venom glands could convert this structure to valinamide, the carboxyl end of secreted $\alpha$-melanotropin (25). A general mechanism for the formation of COOH-terminal amide bonds in peptides is thus a possibility that may warrant further experimentation.

We thank Mrs. Lela Frasel and Dr. Ingela Kindås-Mügge for the supply of venom gland RNA. Dr. D. F. Steiner called our attention to the $\alpha$-melanotropin-corticotropin example.

1. Tuppy, H. & Michl, H. (1953) *Monatsh. Chem.* **84**, 1011–1020.
2. Mutt, V., Jorpes, J. E. & Magnusson, S. (1970) *Eur. J. Biochem.* **15**, 513–519.
3. Bromer, W. W., Sinn, L. G. & Behrens, O. K. (1957) *J. Am. Chem. Soc.* **79**, 2807–2810.
4. Habermann, E. & Jentsch, J. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 37–50.
5. Kreil, G. & Bachmayer, H. (1971) *Eur. J. Biochem.* **20**, 344–350.

6. Kreil, G. (1973) *Eur. J. Biochem.* **33,** 558–566.
7. Kindâs-Mügge, I., Frasel, L. & Diggelmann, H. (1976) *J. Mol. Biol.* **105,** 177–181.
8. Kindâs-Mügge, I., Lane, C. D. & Kreil, G. (1974) *J. Mol. Biol.* **87,** 451–462.
9. Suchanek, G., Kindâs-Mügge, I., Kreil, G. & Schreier, M. H. (1975) *Eur. J. Biochem.* **60,** 309–315.
10. Milstein, C., Brownlee, C. G., Harrison, R. M. & Matthews, M. B. (1972) *Nature New Biol.* **239,** 117–120.
11. Schechter, I. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2256–2260.
12. Kemper, B., Habener, J. F., Mulligan, R. C., Potts, J. T. & Rich, A. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 3731–3735.
13. Boime, I., Boguslawski, S. & Caine, J. (1975) *Biochem. Biophys. Res. Commun.* **62,** 103–109.
14. Devillers-Thiery, A., Kindt, T., Scheele, G. & Blobel, G. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 5016–5020.
15. Sussman, P. M., Tushinski, R. J. & Bancroft, F. C. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 29–33.
16. Chan, S. J., Keim, P. & Steiner, D. F. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1964–1968.
17. Marcu, K. & Dudock, B. (1974) *Nucleic Acid Res.* **1,** 1385–1397.
18. Akabori, S., Ohno, K. & Narita, K. (1952) *Bull. Chem. Soc. Jpn.* **25,** 214–217.
19. Houmard, J. & Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 3506–3509.
20. Haux, P. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350,** 536–546.
21. Haux, P., Sawerthal, E. & Habermann, E. (1967) *Hoppe Seyler's Z. Physiol. Chem.* **348,** 737–743.
22. Callewaert, G. L., Shipolini, R. & Vernon, C. A. (1968) *FEBS Lett.* **1,** 111–113.
23. Scott, A. P., Ratcliffe, J. G., Rees, L. H., Landon, J., Bennett, H. P. J., Lowry, P. J. & McMartin, C. (1973) *Nature New Biol.* **244,** 65–67.
24. Steiner, D. F., Kemmler, W., Tager, H. S. & Peterson, J. D. (1974) *Fed. Proc.* **33,** 2105–2115.
25. Harris, J. I. & Lerner, A. B. (1957) *Nature* **179,** 1346–1347.