Post-translational Processing in Xenopus Oocytes Includes Carboxyl-terminal Amidation*

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Xenopus oocytes are versatile cells capable of carrying out many post-translational processes. Although previously reported not to be capable of C-terminal amidation, this report demonstrates that Xenopus oocytes do indeed have an amidating enzyme. The amidating activity from Xenopus ovaries is compared to the known amidating activity found in porcine pituitaries. The demonstration of C-terminal amidation by Xenopus oocytes extends their usefulness in studying post-translational events.

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Materials and Methods

Live, female Xenopus laevis were obtained from Xenopus Ltd., South Nutfield, Surrey, United Kingdom. Ovarian tissue was removed and used directly (3). Frozen porcine pituitaries were obtained from Walls Meat Co., Hyde, Cheshire, United Kingdom. The Xenopus and porcine tissues were processed identically.

The starting tissue was weighed and then homogenized at 1 g/ml in ice-cold buffer (100 mM NaCl, 25 mM PIPES, pH 6.8, 0.1% Triton X-100, 100 units/ml catalase, 1 mM phenylmethylsulfonyl fluoride). The mixture was centrifuged at 15,000 x g at 4 °C for 20 min. Supernatant was saved at 4 °C and the pellet was rehomogenized in a volume of buffer equal to that used in the first homogenization step. The mixture was centrifuged and the supernatants were pooled. The supernatants were assayed immediately for amidating activity. Each amidating reaction contained 35 μl of tissue homogenate, 5 μl of daasylated D-Tyr-Val-Gly (61 μg/ml), 5 μl of catalase (1.7 x 10^6 units/ml), 5 μl of 10 mM ascorbate, 5 μl of 18 mM Cu(II), and 1 μl of 650 mM KI. The reaction was incubated overnight at 37°C. Volumes of 10 μl were spotted onto a thin layer chromatography plate (HPTLC, RP-18 Fz=S, Merck 13724) and dried, and the chromatography was done at 37 °C in a solvent of methanol/water/dichloromethane (6:3:1). The plates were dried and photographed under long-wave UV light.

The assay for amidating activity is a modification of a published procedure (7, 8). A fluorescent label has been substituted for the radioactive label. To synthesize the fluorescent substrate, a solution of d-Tyr-Val-Gly (1 mg, 2.9 μmol) in sodium bicarbonate (100 μl, 0.2 M) was lyophilized and the residue was dissolved in acetone (500 μl). Distilled water was added (500 μl), and the reaction was incubated at 45°C for 1 h and the products were separated by HPLC (Synchropak RP-P 25 x 1-cm column, 0.1% HCl). One principal product peak was observed and when analyzed by fast atom bombardment mass spectrometry it yielded an ion of m/z 359 corresponding to sodiated dansyl-D-Tyr-Val-Gly. As a control for monitoring the amidating reaction, fluorescent product was also chemically synthesized. A solution of d-Tyr-Val-CONH-HCl (1 mg, 3.2 μmol) in sodium bicarbonate (100 μl, 0.2 M) was dansylated as just described. The HPLC-purified product yielded a protonated fast atom bombardment mass ion at m/z 513 as expected. Using the pure dansyl-d-Tyr-Val-Gly and dansyl-d-Tyr-Val-CONH, methods for separating the product and substrate of the amidating reaction could be evaluated. Rapid and complete separation were obtained using the reverse phase thin layer chromatography plates described above.

Results and Discussion

Crude preparations of homogenized Xenopus ovaries and porcine pituitaries were prepared as outlined under "Materials and Methods." In both preparations the concentration of tissue/volume of homogenization buffer) was kept the same. The frog and porcine extracts were assayed for amidating activity immediately after preparation. The assay employs a synthetic substrate, d-Tyr-Val-Gly, that was originally derived from the known amino acid sequences of α-melanotropin, melittin, and their precursors. This synthetic substrate was also designed to be relatively stable and reasonably resistant to the wide variety of proteolytic enzymes found in
FIG. 1. Assays for amidating enzyme in Xenopus ovaries and porcine pituitaries. Panel A analyzes homogenates of Xenopus ovaries. Panel B analyzes porcine pituitary homogenates. The positions of the dansylated D-Tyr-Val-Gly substrate (S), the dansylated D-Tyr-Val-CONH₂ product (P), and the origin (O) of the TLC plate are indicated. In both panels, lane 1 is a positive control using partially purified porcine pituitary amidating enzyme. lane 2 is a negative control with no enzyme, and lanes 3 to 6 are from 10⁻⁴, 10⁻³, 10⁻², and 10⁻¹ dilutions of the tissue homogenates.

FIG. 2. Assays to determine the temperature sensitivity of amidating enzyme from Xenopus ovaries and porcine pituitaries. As in Fig. 1, the positions of substrate (S), product (P), and origin (O) are indicated. Lanes 1 and 2, undiluted Xenopus ovary homogenate before (lane 1) and after (lane 2) heat treatment (60 °C, 10 min). Lanes 3 and 4, undiluted porcine pituitary homogenate before (lane 3) and after (lane 4) an identical heat treatment. Lane 5 is a positive control using partially purified porcine pituitary amidating enzyme.

The Xenopus ovary and porcine pituitary preparations were assayed for amidating activity using concentrated and diluted preparations. The results are presented in Fig. 1. In both panels, lane 1 is a positive control using partially purified amidating enzyme from porcine pituitaries. Lane 2 in both panels is a negative control where no enzyme was added to the reaction. Arrows on the side point out the positions of the substrate (S) and product (P). Panel A, lanes 3 to 6, presents the results analyzing the Xenopus preparation at 10⁻⁴, 10⁻³, 10⁻², and 10⁻¹ dilutions, respectively. Using the concentrated Xenopus preparation, a substantial proportion of the substrate was converted to product (Fig. 1A, lane 3). When the preparation was diluted 10-fold, a small amount of product was still formed (Fig. 1A, lane 4). Clearly, there is an amidating activity in Xenopus ovaries.

Fig. 1B, lanes 3 to 6, presents the results analyzing the porcine preparation at 10⁻⁴, 10⁻³, 10⁻², and 10⁻¹ dilutions, respectively. Using the concentrated porcine preparation, virtually all of the substrate was converted to product (Fig. 1B, lane 3). Even a 100-fold dilution of the porcine preparation could give a significant amount of product formation (Fig. 1B, lane 5). As expected, the porcine pituitary preparation contained high levels of amidating activity.

Thus, both Xenopus ovaries and porcine pituitaries contain amidating enzyme. Quantitative comparisons are difficult from such crude preparations, but the original tissues were homogenized in equal volumes of buffer with respect to the tissue wet weight. By comparing the amount of amidating activity per gram of original tissue, porcine pituitaries are estimated to contain 20 to 50 times more amidating activity than Xenopus ovaries (see Fig. 1).

Concentrated preparations from both Xenopus ovaries and porcine pituitaries were tested for thermolability of the amidating enzymes. Amidating enzyme from porcine pituitary is relatively stable at 60 °C.² Both preparations were heated to 60 °C for 10 min. Concentrated preparations were then assayed before and after heat treatment. The heat treatment had no detectable effect on amidating activity from porcine pituitaries (Fig. 2, lane 3 and 4). With the preparation from Xenopus ovaries, however, the heat treatment destroyed most, if not all, of the amidating activity. The reason for this difference in thermostability between amidating enzyme from Xenopus ovaries and porcine pituitaries is not known. It does suggest that the amidating activities observed in the two tissues are not due to identical enzymes.

The tissue distribution of amidating enzyme is not well known (2). It is assumed that amidating enzymes will be found in a variety of tissues involved in the amidation of peptide hormones (8). These peptides are widely distributed in neural and endocrine tissues. The presence of amidating enzyme has been reported in pituitary, thyroid, pancreas, hypothalamus, submandibular glands, cerebrospinal fluid, and serum (1, 9, 10). Xenopus ovarian tissue, as well as individual mature oocytes (data not presented), have now been demonstrated to have amidating enzyme. Besides extending the range of tissues demonstrated to have amidating enzyme, knowing that Xenopus oocytes are capable of C-terminal amidation broadens their potential usefulness in studying post-translational events.

²D. Low, personal communication.
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REFERENCES
1. Bradbury, A. F., and Smyth, D. G. (1986) in Biogenetics of Neurohormonal Peptides, pp. 171–186, Academic Press, London
2. Kreil, G. (1985) in The Enzymology of Post-translational Modification of Proteins, Vol 2, pp. 41–51, Academic Press, London
3. Colman, A. (1984) in Transcription and Translation: A Practical Approach, (Hames, B. D., and Higgins, S. J., eds) pp. 271–302, IRL Press Ltd., Oxford and Washington D. C.
4. Kindas-Mugge, I., Lane, C. D., and Kreil, G. (1974) J. Mol. Biol. 87, 451–462
5. Lane, C. D., Champion, J., Haimi, L., and Kreil, G. (1981) Eur. J. Biochem. 113, 273–281
6. Suchanek, G., and Kreil, G. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 975–978
7. Bradbury, A. F., Finnie, M. D. A., and Smyth, D. G. (1982) Nature 298, 686–688
8. Bradbury, A. F., and Smyth, D. G. (1985) Biochem. Biophys. Res. Commun. 112, 372–377
9. Eipper, B. A., Myers, A. C., and Mains, R. E. (1985) Endocrinology 116, 2497–2504
10. Wand, G. S., Ney, R. L., Mains, R. E., and Eipper, B. A. (1985) Neuroendocrinology 41, 482–489