Enzymic Cleavage of Post-Proline Peptide Bonds: Degradation of Arginine-Vasopressin and Angiotensin II[1] (36796)

HADARA SHLANK AND RODERICH WALTER

Department of Physiology, The Mount Sinai Medical and Graduate Schools of the City University of New York, New York, New York 10029

An endopeptidase with a high degree of specificity for the hydrolysis of peptide bonds at the carboxyl side of proline would be a desirable candidate in general for the selective degradation of peptides and proteins, and would be of value for sequence and structure analysis in particular. Such a proteolytic enzyme has to the best of our knowledge not been previously reported. However, while studying the inactivation of the neurohypophyseal hormone, oxytocin, we noted that a partially purified enzyme from human uteri released the dipeptide leucylglycinamide by specifically hydrolyzing the Pro^7-Leu^8 peptide bond of the hormone (1).

In the present communication we describe a further purification of this uterine enzyme and its mode of degrading the two proline-containing hormones, arginine-vasopressin and angiotensin II (Fig. 1).

Materials. The unlabeled arginine-vasopressin (AVP) used was that prepared by Meienhofer *et al.* (2). [9-^{14}C-Glycinamide]vasopressin ([^{14}C]AVP) and [9-^{14}C-glycinamide]oxytocin ([^{14}C]OXY) were prepared by Walter and Havran (3); these labeled hormones, which have retained full biological activities (3), had been stored at 4° dissolved in 0.25% acetic acid containing 0.1% chlorobutanol or in glass-distilled water, respectively. Ile^5-Angiotensin II (grade A) and dithiothreitol (DTT) were purchased from Calbiochem.

Enzyme preparation. Human uterine tissue was homogenized in 0.01 *M* sodium phosphate buffer, pH 6.5, for 1 min. The homogenate was centrifuged at 105,000*g* for 20 min, and the supernatant was chromatographed on a DEAE-cellulose column as previously described (1, 4). The protein peak eluted between 0.04–0.06 *M* phosphate buffer was pooled, DTT was added to a final concentration of 0.5 m*M*, and the protein was precipitated by dialysis for 5–8 hr against 20 vol of 80% $(NH_4)_2SO_4$ containing 0.5 m*M* DTT. The precipitate was dissolved in 25 m*M* sodium phosphate buffer (pH 6.5) containing 1 m*M* EDTA and 0.5 m*M* DTT, and the solution was subjected to gel filtration on a Sephadex G-100 column using the same buffer (Fig. 2). All the above procedures were carried out at 4°.

[^{14}C]OXY was used as a marker to locate and to determine the degree of separation between two enzymic activities, one capable of releasing Leu-Gly-NH_2 and the other Gly-NH_2 from the hormone. Aliquots of alternate fractions of eluate were tested for enzymic activity by incubation for 15 min at 37° with 5 μg [^{14}C]OXY. The products released, H-Leu-Gly-NH_2 and/or H-Gly-NH_2, were identified by electrophoresis, as previously described (1). Those fractions releasing H-Leu-Gly-NH_2 from oxytocin (fractions 26–30) were pooled; aliquots were kept frozen for subsequent studies. This is referred to as the enzyme preparation.

Enzyme degradation of AVP. A mixture of 400 μg of AVP and 2 μg of [^{14}C]AVP was incubated overnight at 37° in 1 ml of sodium phosphate buffer (final conc 0.05 *M*, pH 7.4) with 1 mg of human uterine enzyme protein, determined according to the method of Wahrburg and Christian (5). The reaction was terminated by a 5-min heat treatment. The enzyme digest was lyophilized, redissolved in 0.1 ml of water, and subjected to high-voltage electrophoresis using 0.5% pyri-

[1] This work was supported in part by the U.S. Public Health Service Grant AM-13567.

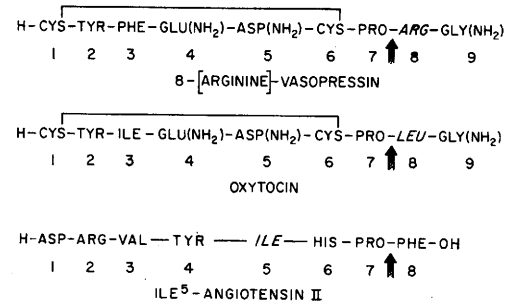

FIG. 1. Primary structure of arginine-vasopressin, oxytocin and Ile^5-angiotensin II. Numbers indicate positions of amino acid residues. Arrows depict the sites of hydrolytic cleavage by human uterine enzyme preparation.

dine–5% acetic acid buffer (pH 3.5, 1500 V, 3–4 mA/cm, 2 hr). AVP (incubated with buffer alone as a control), H-Pro-Arg-Gly-NH_2 and glycinamide served as markers. Areas of the electrophoretogram which contained ninhydrin-active or radioactive material (detected and measured with a Baird Atomic Scanogram II) were eluted with 10% acetic acid and lyophilized. One portion of this material was subjected to amino acid analysis (6) on a Beckman amino acid analyzer (model 121C) and another was hydrolyzed for 22 hr at 110° with 6 *N* HCl and then subjected to amino acid analysis.

Relative rates of release of the C-terminal dipeptide from labeled AVP and oxytocin. In independent experiments, 5 μg of [^{14}C]AVP or [^{14}C]OXY were incubated with 0.1 mg of enzyme protein in a final volume of 0.5 ml sodium phosphate buffer (0.05 *M*, pH 7.4) at 37° for time intervals of 0, 5, 10, 30, and 60 min. The reactions were stopped by heat treatment. The digest constituents were separated electrophoretically, and amounts of C-terminal dipeptide released from the hormones were determined with a chromatogram scanner, as described above.

Enzyme degradation of Ile^5-angiotensin II. One milligram of hormone was incubated at 37° with 0.5 to 0.6 mg of enzyme protein in a final volume of 1 ml of 0.05 *M* sodium phosphate buffer, pH 7.4. Samples (200 μl each) were withdrawn after 0, 30, 60, 120, and 240 min of incubation, and were immediately heated at 100° for 5 min to inactivate enzyme. Half of each heat-denatured sample was subjected to amino acid analysis and the other half to electrophoretic separation as described above, except that 2000 V were used. Hormone incubated with buffer alone and Phe served as markers.

Results and Discussion. Previously we reported on an enzyme preparation (obtained from human uteri by DEAE chromatography) which rapidly inactivated oxytocin by cleaving the Pro^7-Leu^8 peptide bond (1). Certain fractions of that enzyme preparation, however, showed overlap of activity catalyzing H-Gly-NH_2 release and activity catalyzing H-Leu-Gly-NH_2 release (1). In the present study these activities have been separated for all practical purposes by gel filtration on Sephadex G-100. The activity catalyzing release of H-[1-^{14}C]Gly-NH_2 from

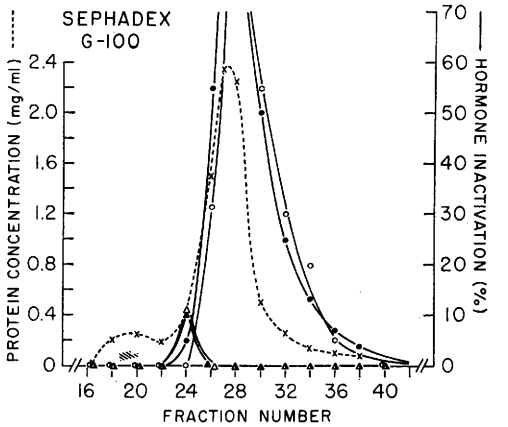

FIG. 2. Purification of human uterine enzyme by Sephadex G-100 gel filtration. Approximately 60 mg of an ammonium sulfate precipitate of the DEAE-fractionated human uterine enzyme (eluate between 0.04 and 0.06 *M* sodium phosphate buffer, pH 6.5) were dissolved in 5 ml of 25 m*M* sodium phosphate buffer containing 1 m*M* EDTA and 0.5 m*M* DTT. The mixture was applied to a Sephadex G-100 column (85 × 2 cm) equilibrated with the above buffer, and then developed with the same buffer. The eluate was collected in three 5-ml fractions/hr. Aliquots of alternate fractions were tested by UV-absorption for protein content (X) and for their capability to cleave radioactive H-Leu-Gly-NH_2 (●), or H-Gly-NH_2 (▲) from [^{14}C]oxytocin (which served as a marker for the purification), and radioactive H-Arg-Gly-NH_2 (○) or H-Gly-NH_2 (△) from [^{14}C]AVP.

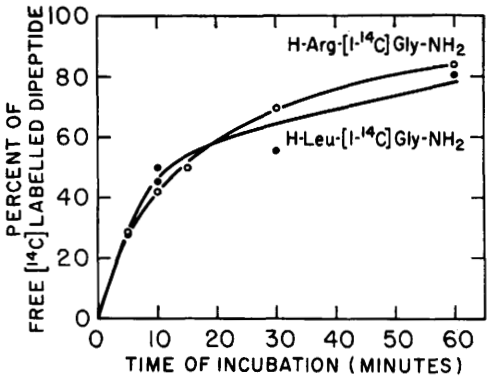

Fig. 3. Rates of release of COOH-terminal dipeptides from radioactively labeled arginine-vasopressin and oxytocin by human uterine enzyme preparation. Incubation procedure and electrophoretic separation of digest mixture are detailed in the text.

oxytocin was found in fractions 22–25, whereas that catalyzing release of H-Leu-[1-^{14}C]Gly-NH_2 was present in fractions 26–30 (see Fig. 2). The pH optimum of the latter enzyme, the post-proline cleaving enzyme, was 7.4 as determined in sodium phosphate buffer. As the purity of the enzyme increased, DTT was required to stabilize its activity. Precipitation of the enzyme with $(NH_4)_2SO_4$ followed by gel filtration of the redissolved precipitate led in the absence of DTT to a 90–95% loss of activity. However, the activity was fully restored upon addition of 0.5 m*M* DTT, which was therefore added to the enzyme preparation. Because different enzymes can be delineated not only by their substrate specificity but also by their activation behavior, we studied the effect of Mg^{2+} and Mn^{2+} on the post-proline cleaving enzyme. Concentrations of these divalent cations ranging from 10^{-6} to 10^{-3} *M* had no detectable effects on the specific activity of the enzyme in releasing H-Leu-Gly-NH_2 from oxytocin.

When aliquots of fractions eluted from the Sephadex G-100 were incubated with [^{14}C]AVP, the distribution pattern of the antidiuretic hormone-inactivating activity was essentially identical with that obtained for [^{14}C]OXY (Fig. 2). Moreover, the rates of inactivation of labeled OXY and AVP were identical when incubated for as long as 60 min with uterine enzyme preparation (Fig. 3).

We then determined the cleavage products resulting from the treatment of AVP with the enzyme preparation. One aliquot of the digest was subjected directly to amino acid analysis to test for the possible release of any free amino acids; none were detected. A second aliquot of digest was then applied to electrophoretic separation as described by Koida *et al.* (7) and Walter *et al.* (4). Three well-separated, major radioactive peaks were detected on the electrophoretogram, at sites 12, 18, and 26 cm from the origin. The slowest-moving component corresponded in its electrophoretic mobility to AVP, whereas the labeled compound with the highest electrophoretic mobility was identified as H-Arg-Gly-NH_2, based on amino acid analysis after elution from the electrophoretogram, acidolysis, and repeated lyophilization (molar ratios of ninhydrin-active components with glycine taken as 1.0 were: Arg 0.8; Gly 1.0; and ammonia 1.2). The component with intermediate electrophoretic mobility was judged to be the diketopiperazine of the C-terminal dipeptide, based on the amino acid composition (ratios: Gly 1.00 and Arg 1.05), the reduced electrophoretic mobility compared to H-Arg-Gly-NH_2 and finally, on the finding that this material increases with time as the amount of H-Arg-Gly-NH_2 decreases. None of the radioactive peaks corresponded to the electrophoretic mobility of H-Gly-NH_2 (30 cm) or H-Pro-Arg-Gly-NH_2 (23 cm). These data clearly show that the human uterine enzyme hydrolyzes the peptide bond on the carboxyl side of the proline residue in AVP.

We next incubated the enzyme preparation with the hormone Ile^5-angiotensin II which like AVP contains a single proline residue. Electrophoretic separation of the hormone–enzyme digest mixture revealed only two ninhydrin-positive bands, located at 5 and 17 cm from the origin. The first band was identified as Phe, by comparison with the migration position of authentic Phe, and this was later confirmed by amino acid analysis of the digest mixture. The second band corresponded in its mobility to Ile^5-angiotensin II, and was thought to be comprised of a

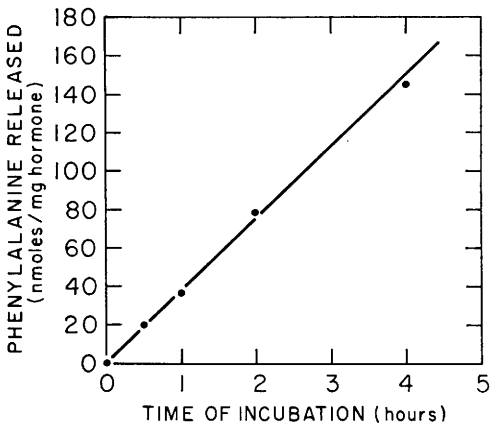

FIG. 4. Release of phenylalanine as a function of time from Ile^5-angiotensin II by human uterine enzyme preparation. For incubation procedure and methods of analysis of the digest mixture by electrophoresis and amino acid analysis, see text.

mixture of undegraded hormone and of des-Phe-angiotensin II. The amount of Phe released increased linearly with time over an incubation period of at least 240 min (Fig. 4). The results are expressed as nanomoles of Phe released per milligram of Ile^5-angiotensin II.

These data clearly show that the partially purified human uterine enzyme degrades not only oxytocin (1) but also AVP and Ile^5-angiotensin II, in each instance hydrolyzing the hormonal peptide bond on the carboxyl side of a proline residue. The enzymic characteristics of this preparation differ in several respects from those reported for angiotensin-inactivating, "lysosomal" enzymes from kidney, liver or other tissues (8–11). The "lysosomal" enzyme was reported to be optimally active at pH 5.7 and, though exhibiting specificity for cleaving the peptide bond at the carboxyl side of proline residues, this carboxypeptidase catalyzes the process only if the proline residue is located at the penultimate position of the peptide. In contrast, the human uterine enzyme was optimally active at pH 7.4, and cleaved peptide bonds at the carboxyl side of proline residues independent of the location of the residue, as revealed not only by the inactivation of angiotensin, but also of AVP and OXY.

Further characterization of properties and specificity of the human uterine enzyme is warranted.

Summary. A partially purified enzyme from human uterine tissue was found to cleave peptides specifically at the carboxyl side of proline residues when arginine-vasopressin, oxytocin, and Ile^5-angiotensin II were tested as proline-containing substrates. The enzyme has a pH optimum of 7.4; its activity is not affected by Mn^{2+} or Mg^{2+} (10^{-6} to 10^{-3} *M*).

The authors thank Dr. T. D. Kerenyi for his active participation and interest in this work. The technical help of Mrs. I. Mintz was appreciated.

1. Walter, R., Shlank, H., Glass, J. D., Schwartz, I. L., and Kerenyi, T. D., Science **173**, 827 (1971).

2. Meienhofer, J., Trzeciak, A., Havran, R. T., and Walter, R., J. Amer. Chem. Soc. **92**, 7199 (1970).

3. Walter, R., and Havran, R. T., Experientia **27**, 645 (1971).

4. Walter, R., Glass, J. D., Dubois, B. M., Koida, M., and Schwartz, I. L., *in* "Proc. 2nd American Peptide Symposium, Cleveland, 1970" (S. Lande, ed.), p. 17, 327 (1972). Gordon and Breach, London.

5. Wahrburg, O., and Christian, W., Biochem. Z. **310**, 384 (1941).

6. Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem. **30**, 1190 (1958).

7. Koida, M., Glass, J. D., Schwartz, I. L., and Walter, R., Endocrinology **88**, 633 (1971).

8. Yang, H. Y. T., Erdös, E. G., and Chiang, T. S., Nature (London) **218**, 1224 (1968).

9. Yang, H. Y. T., Erdös, E. G., Chiang, T. S., Jenssen, T. A., and Rogers, J. G., Biochem. Pharmacol. **19**, 120 (1970).

10. Matsunaga, M., Kira, J., Saito, N., Ogino, K., and Takayasu, M., Jap. Circ. J. **32**, 137 (1968).

11. Matsunaga, M., Jap. Circ. J. **35**, 333 (1971).

Received Apr. 26, 1972. P.S.E.B.M., 1972, Vol. 141.