*ENZYMATIC INACTIVATION OF PEPTIDE HORMONES POSSESSING A C-TERMINAL AMIDE GROUP**

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Abstract.—A partially purified enzyme extracted from the bladder of the toad, *Bufo marinus* L., was found to cleave the glycine amide moiety from oxytocin, 8-lysine-vasopressin, 8-arginine-vasopressin, and other hormone analogs terminating in a primary carboxamide group; however, this enzyme does not attack hormone analogs terminating with a methylamide, dimethylamide, or carboxyl group. Preliminary experiments indicate that a functionally similar enzyme is also present in the mammalian kidney, the major target organ of neurohypophyseal antidiuretic hormones. This enzyme, besides inactivating oxytocin and 8-lysine-vasopressin, also cleaves the phenylalanine amide moiety from a tetrapeptide analog of gastrin, another hormone terminating in a primary carboxamide group. Attention is drawn to the possible general significance of "carboxamidopeptidases" for the termination of the action of peptide hormones in which the C-terminal amino acid residue bears a carboxamide group.

A mode of inactivation of neurohypophyseal hormones specific for terminating their physiological action has not been established, although a number of enzymes capable of degrading these peptides have been studied.[1]

Recently we reported on the partial purification from the gravid rat uterus of an enzyme which was found to catalyze the release of glycine amide from the C-terminus of oxytocin.[2] The substrate specificity of this uterine protease, which attacks a conspicuous structural element of the hormone, in conjunction with the finding of a qualitatively altered uterotonic response to an oxytocin analog (9-decarboxamido-oxytocin) lacking the terminal glycine amide,[3] suggested to us that this enzyme may be a physiologically significant regulator of hormone action. For this reason we have not limited our investigation of such enzymatic activity to a single neurohypophyseal hormonal target organ but, in fact, have extended this study to the amphibian bladder and mammalian kidney; the preliminary results are reported below.

To explore the specificity of such enzyme systems, we studied neurohypophyseal hormones and analogs, which can be categorized in three groups (see Table 1). The first group of these compounds is resistant to amino peptidase action by virtue of a deletion of the N-terminal amino group (deamino analogs); the second group consists of hormonal peptides with structural variations of the amino acid residue in position 8; and the third group consists of analogs with substitutions of the amide of the glycine residue in position 9.

The enzyme from the bladder of the toad, *Bufo marinus* L., was prepared by the method of Campbell, Thysen, and Chu,[4] who had already reported that it releases the C-terminal amino acid of 8-lysine-vasopressin. Extract (1 ml) con-

TABLE 1. *Neurohypophyseal peptides studied for their susceptibility to inactivation by partially purified enzyme of the amphibia bladder.*[a]

	1	2	3	4	5	6	7	8	9
Oxytocin[b]	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	$GlyNH_2$
Oxytocinoic acid[c]	—	—	—	—	—	—	—	—	GlyOH
3-Phenylalanine-oxypressin[d]	—	—	Phe	—	—	—	—	Ala	—
Deamino-3-phenylalanine-oxypressin[d]	βMpr	—	Phe	—	—	—	—	Ala	—
Deamino-oxytocinoic acid methylamide[e]	βMpr	—	—	—	—	—	—	—	$GlyNHCH_3$
Deamino-oxytocinoic acid dimethylamide[e]	βMpr	—	—	—	—	—	—	—	$GlyN(CH_3)_2$
8-Lysine-vasopressin[f]	—	—	Phe	—	—	—	—	Lys	—
8-Arginine-vasopressin[g]	—	—	Phe	—	—	—	—	Arg	—

[a] Neurohypophyseal hormones are denoted in accordance with the IUPAC-IUB Tentative Rules (*Biochemistry*, **6**, 362 (1967)). Standard abbreviations are used for amino acid residues (*Biochemistry*, **5**, 2485 (1966)); in addition, βMpr stands for β-mercaptopropionyl. The amino acids (except glycine) are of the L-configuration.

[b] du Vigneaud, V. C. Ressler, J. M. Swan, C. W. Roberts, P. G. Katsoyannis, and S. Gordon, *J. Am. Chem. Soc.*, **75**, 4879 (1953); cf. Manning, M., *J. Am. Chem. Soc.*, **90**, 1348 (1968).

[c] Ferrier, B. M., and V. du Vigneaud, *J. Med. Chem.*, **9**, 55 (1966).

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[e] Takashima, H., W. Fraefel, and V. du Vigneaud, *J. Am. Chem. Soc.*, **75**, 6182 (1969).

[f] Cf. Meienhofer, J., and Y. Sano, *J. Am. Chem. Soc.*, **90**, 2996 (1968).

[g] Cf. Huguenin, R. L., and R. A. Boissonnas, *Helv. Chim. Acta*, **45**, 1629 (1962).

taining the enzyme from $1^1/_2$ toad hemibladders was incubated with 0.1 ml of a 10^{-3} *M* substrate solution at 37° for four hours (incubates with enzyme and substrate alone were included as control throughout the entire experiment). The precipitated protein, obtained by treatment of incubate with 0.1 ml of 5 per cent trichloroacetic acid, was removed by centrifugation. The deproteinized incubate was evaporated to dryness under reduced pressure in the presence of KOH and P_2O_5, and the resulting residue was examined for enzymatic degradation product(s) as follows: an aliquot of the incubate was treated with 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl-Cl) according to a modified procedure of Gray and Hartley.[5] One portion of the reaction mixture was analyzed by thin-layer chromatography directly, while another portion was subjected to hydrolysis with 6 *N* HCl for eight hours at 100°, evaporated to dryness, and then developed by thin-layer chromatography. The dansylated incubate and the standard amino acid derivatives were compared by using three chromatographic systems: (1) cellulose layer developed with 0.8 per cent acetic acid containing 0.4 per cent pyridine[6]; (2) silica gel-G layer with chloroform-methanol-acetic acid (70:30:3, v/v/v); and (3) the silica gel-G layer with chloroform-isoamyl alcohol-acetic acid (70:20:5, v/v/v). The cellulose plates were allowed to air-dry thoroughly before being viewed, whereas the silica plates were examined immediately after chromatography. In the case of neurohypophyseal peptides that possess an N-terminal primary amino group, another aliquot of the desiccated incubate was oxidized with performic acid[7] prior to dansylation and chromatographic analysis on cellulose layers with the solvent system butanol-acetic acid-water (6:2:2, v/v/v). A sample of cystine was carried through the oxidation procedure and all subsequent steps. Glycine methylamide hydrochloride[8]

and glycine dimethylamide picrate[9] were dansylated to serve as standards during chromatographic analyses of experiments involving deamino-oxytocinoic acid methylamide and deamino-oxytocinoic acid dimethylamide.

It was found that the enzyme from the toad bladder releases glycine amide from both oxytocin and 8-lysine-vasopressin. Unlike chymotrypsin which inactivates oxytocin but not 8-lysine-vasopressin,[10] and unlike trypsin which inactivates 8-lysine-vasopressin but not oxytocin,[11, 12] this enzyme inactivates both oxytocin and 8-lysine-vasopressin. In addition, it was established that neurohypophyseal hormonal peptides do not require an N-terminal amino group to be recognized as substrates by this enzyme. It was also noted that an isoleucine or phenylalanine residue in position 3 did not affect the release of glycine amide from the hormonal peptides. Of greater importance is the fact that the side chain of the amino acid residue in position 8 also seems to play no decisive role in determining glycine amide release. However, for the protease to recognize a neurohypophyseal peptide as a substrate the carboxamide group in position 9 proved to be critical. While the enzyme readily attacks 8-lysine-vasopressin, 8-arginine-vasopressin, oxytocin, and other hormone analogs terminating in a primary carboxamide moiety, only traces of glycine methylamide and glycine dimethylamide were detected upon incubation with the enzyme of deamino-oxytocinoic acid methylamide and deamino-oxytocinoic acid dimethylamide; in the latter two neurohypophyseal hormone analogs, one or both of the amide hydrogen atoms have been replaced by methyl groups. Oxytocinoic acid—an analog in which the carboxamide moiety in position 9 has been replaced by a carboxyl group—was essentially unaffected by the enzyme, even with high enzyme-hormone ratios or prolonged incubation times.

The foregoing results as well as general considerations (*vide infra*) suggest that this type of enzyme, which we refer to as "carboxamidopeptidase," may be a crucial physiological modulator which terminates the action of neurohypophyseal hormones in their various target organs. Moreover, it is likely that an enzyme system, possessing a substrate specificity similar or identical to the substrate specificity of the "carboxamidopeptidase" described in this study, is important in the inactivation and, therefore, in the over-all regulation of action of a defined class of peptides—e.g., gastrin,[13] secretin,[14] caerulein,[15] phyllocaerulein,[16] pancreozymin-cholecystokinin,[17] and thyrocalcitonin[18]—in which the C-terminal amino acid residue carries a carboxamide rather than a free α-carboxyl group, thus rendering these molecules unsusceptible to the action of carboxypeptidases. In this context it is noteworthy that Laster and Walsh,[19] while surveying various rat tissues for the presence of amidase activity, which releases NH_3 from a protected C-terminal tetrapeptide of gastrin, observed that a component of kidney tissue catalyzes the hydrolysis of the peptide bond between aspartic acid and phenylalanine amide. Since the kidney is the major target organ of the vasopressins, we investigated rat kidney homogenate for enzymatic activity directed at the C-terminal amino acid amide of neurohypophyseal hormones.

One gram of kidney tissue from male Sprague-Dawley rats (wt $\sim$200 gm) was homogenized in 10 ml of deionized water at 0°. The homogenate was centrifuged at 110,000 g for 20 minutes at 4° and the sediment discarded. The centrifuged

supernatant was applied to an 0.8-gm bed of diethylaminoethyl cellulose which had been previously equilibrated with 0.1 *M* potassium phosphate pH 7.6 and then washed with water. The column was developed successively with water, 0.1 *M* sodium phosphate pH 6.5, and then with 0.1 *M* sodium phosphate pH 6.5 containing 1 *M* sodium chloride. Effluent from the column was monitored continuously for ultraviolet absorption (254 mμ) and collected in 5-ml quantities. Fractions from the three ultraviolet-absorbing regions were examined for oxytocinase activity by the method described earlier for rat uterine enzyme,[2] except that column fractions were diluted 1:4 with 0.1 *M* sodium phosphate pH 6.5 before assay. Fractions which destroyed the uterotonic effect of oxytocin were incubated with 0.1 ml of a 10^{-3} *M* oxytocin solution for examination of degradation products according to the procedures detailed in the section dealing with the toad bladder preparation. Although all three fractions degraded oxytocin readily, only the fraction eluted with 0.1 *M* sodium phosphate containing 1 *M* sodium chloride gave glycine amide as a clearly identifiable product of the hormone inactivation. The finding that 8-lysine-vasopressin was inactivated analogously to oxytocin indicates that this mammalian target organ contains an enzyme system functionally similar to the carboxamidopeptidase from the amphibian bladder.

As indicated above, we feel that carboxamidopeptidase activity may play a role in the inactivation not only of neurohypophyseal hormones but also of the growing category of peptide hormones known to terminate in an amino acid amide. Hence, we extended our studies with the rat kidney enzyme to L-tryptophyl-L-norleucyl-L-aspartyl-L-phenylalanine amide; this tetrapeptide which was obtained after the liberation of the N-terminal amino group of BOC-L-tryptophyl-L-norleucyl-L-aspartyl-L-phenylalanine amide, previously prepared in this laboratory,[20] is an analog of gastrin. Preliminary experiments have indicated that phenylalanine amide is released from this free hormonal analog.

The hypothetical physiological significance of this carboxamidopeptidase system warrants further study. For example, we should like to know whether the presence of this enzyme is limited to target organs for peptide hormones with terminal carboxamide groups. Further purification of this enzyme(s) is necessary for kinetic studies. In a more practical manner, we plan to investigate the possible role of this enzyme in the perpetuation of normal pregnancy and its potential for the prevention of premature labor.

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