RAT LIVER ENZYME INACTIVATING OXYTOCIN AND ITS DEAMINO-CARBA ANALOGUES

L.Fruhaufová, E.Suska-Brzezińska*, T.Barth and I.Rychlík

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received March 15th, 1973

An enzyme capable of inactivating oxytocin and its deamino-carba analogues was isolated in a partially purified form from rat liver homogenate. The procedure including ionex chromatography, precipitation by ammonium sulfate and gel filtration resulted in 150 fold purification of the enzyme. Kinetic studies showed that the affinity of the enzyme to oxytocin and its deamino-carba analogues is approximately the same. Paper chromatography revealed the release of glycineamide from the linear peptide chain of deamino-dicarba[1,6]-oxytocin.

At present the main attention of studies on the metabolism of neurohypophysical hormones is aimed at enzymes which split the peptide bonds in the linear part of the peptide chain. Both in oxytocin and in vasopressins, the linear chain is formed by three amino acids, the oxytocin sequence being Pro-Leu-GlyNH_2 and that of lysine- and arginine-vasopressin Pro-Lys-GlyNH_2 and Pro-Arg-GlyNH_2, respectively. An enzyme capable of splitting the bond between proline and the following amino acid has as yet been found only in the human uterus; it releases Leu-Gly.NH_2 from oxytocin and Arg-GlyNH_2 from vasopressin[1]. However, an enzymic system which releases the terminal glycineamide seems to be more widely distributed. In this way, vasopressins are split by trypsin[2] and trypsin-like enzymes[3], and oxytocin by chymotrypsin[4]. It was assumed that enzymes capable of releasing glycineamide from oxytocin and vasopressin are present in many tissues. Carboxyamidases, as these enzymes were named, were isolated from the rat uterus[5] and kidney[6], and it was suggested that they are also present in the liver[7].

Like the products of aminopeptidase cleavage[8], the products of carboxyamidase (or chymotrypsin and trypsin) cleavage — de-9GlyNH_2-oxytocin and de-9GlyNH_2-vasopressin — are practically devoid of biological activity on the smooth muscle, blood pressure and rate of urine flow. However, it was shown that the products of carboxyamidase cleavage might participate in metabolic processes in the brain influencing memory consolidation[9].

The enzymic cleavage of the linear chain of the peptide molecule is probably the only possible way in which the analogues of oxytocin and vasopressin, protected against reduction and aminopeptidase cleavage, are inactivated. In our earlier work[7], we established that deamino-carba analogues of oxytocin are inactivated at a lower rate than oxytocin in rat uterine, liver and kidney homogenates. Nevertheless, the

* Visiting scientist from the Department of Animal Physiology, Faculty of Agriculture, Cracow, Poland.

analogues were inactivated and this fact led us to isolate the enzyme responsible for the cleavage of the peptides in the liver. In the present work we describe the partial purification of the enzyme and the cleavage of oxytocin and some of its analogues.

EXPERIMENTAL

Material. Oxytocin was a commercial preparation of Spofa, Prague. The deamino-carba analogues of oxytocin* were prepared[10,11] at the Department of Organic Synthesis of this Institute. Sephadex G 100, DEAE-Sephadex and CM-Sephadex were purchased from Pharmacia, Uppsala. Female rats of the Wistar-Konárovice strain, weighing 180—200 g, were used in experiments.

Determination of enzyme activity. Determination of protein concentration was performed according to Lowry et coworkers[12] or by measuring the absorbance at 280 nm. For measuring the enzyme activity in all extracts and purification fractions, an aliquot of the enzyme preparation was incubated with the substrate for 0—90 min at 37°C. The incubation mixture had the following composition: $2{\cdot}5 \cdot 10^{-7}$—$4 \cdot 10^{-6}$M substrate, $5 \cdot 10^{-2}$M sodium phosphate buffer (pH 7·4) and 0·1—0·5 mg of protein/ml. The reaction was terminated by heating for 3 min in boiling water and the residual hormonal activity was determined on the isolated rat uterus according to Munsick[13].

Purification of oxytocin-inactivating enzyme. The liver homogenate and its 105 000 *g* supernatant were prepared by a method described earlier[7]. All the purification steps were performed at 0—4°C. CM-Sephadex C 50, which had been regenerated through an acid-base cycle and equilibrated with 0·01M ammonium acetate (pH 7·0), was packed in a $15 \times 2{\cdot}5$ cm column. The 105000 *g* supernatant of liver homogenate was passed through the column and eluted with 0·01M ammonium acetate (pH 7·0). The inactivating enzyme was not adsorbed on the column. A column of DEAE-Sephadex A 50 (40×3 cm), which had been regenerated through an acid-base cycle, was equilibrated with 0·02M sodium phosphate buffer (pH 7) containing $1 \cdot 10^{-6}$M-EDTA. The material eluted from the column of CM-Sephadex was applied to the column of DEAE-Sephadex and the unadsorbed material was washed out with 0·02M sodium phosphate buffer containing $1 \cdot 10^{-6}$M-EDTA. The column was then eluted with a linear NaCl gradient (0—0·5M-NaCl in the same buffer). The enzymically active fractions were combined and dialysed 36 h against $1 \cdot 10^{-6}$M-EDTA. Solid ammonium sulphate was then added to the dialysed preparation in an amount resulting in 50% concentration. The solution was stirred (1 h) and the precipitate was removed by centrifugation at 3000 *g*. The concentration of ammonium sulphate in the supernatant was then increased to 70% and the mixture was stirred for 1 h. After centrifugation the precipitate was redissolved in $1 \cdot 10^{-6}$M-EDTA and dialysed against the same solution. The enzyme preparation was used for investigating the inactivation rate of oxytocin and its analogues. In some cases the material was subjected to further purification on a 2×60 cm column of Sephadex G 100 equilibrated with 0·01M sodium phosphate buffer (pH 7) containing $1 \cdot 10^{-6}$M-EDTA. The material was applied to the column and eluted with the same buffer at a rate of 6 ml per hour.

Identification of cleavage products. An aliquot of samples obtained by the incubation of the purified enzyme with DDCOT was taken to dryness, redissolved in 0·1 ml of distilled water

* Abbreviations: DCOT-1, α-deamino-cystathionine^{6,1}-oxytocin (deamino-carba^1-oxytocin); DCOT-6, β-deamino-cystathionine^{1,6}-oxytocin (deamino-carba^6-oxytocin); DDCOT, α-aminosuberic acid^1-oxytocin (deamino-dicarba^{1,6}-oxytocin).

and applied to a sheet of Whatman No 3 chromatographic paper. After descending chromatography in a solvent system composed of n-butanol–pyridin–acetic acid–water (15 : 10 : 3 : 12) with DDCOT and glycineamide as standards, the chromatogram was developed in a 0·5% ninhydrin solution in acetone and fixed in 0·1% $Cu(NO_3)_2$.

RESULTS

Purification Procedure

The 105 000 *g* supernatant which represents a crude extract of rat liver homogenate was freed of some inactive proteins by passing it through a column of CM-Sephadex in equilibrium with 0·01M ammonium acetate which was also used for elution. The enzymically active protein fraction was not adsorbed on the column but was recovered almost completely in the eluate. This step resulted in only slightly higher specific enzyme activity but proved useful in preserving the activity of the inactivating enzyme in the further purification steps. The addition of EDTA in $1 . 10^{-6}$M concentration to all the solutions used in the further purification procedure also helped prevent greater losses of enzyme activity.

The inactivating enzyme eluted from the column of CM-Sephadex was applied to a column of DEAE-Sephadex in equilibrium with 0·02M sodium phosphate

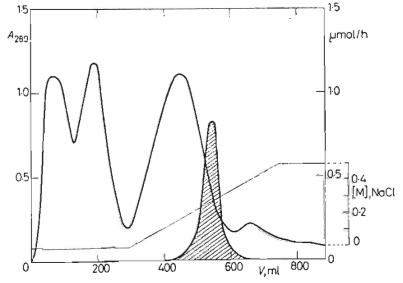

FIG. 1

Chromatography on DEAE-Sephadex

20 ml of enzymically active eluate (660 mg of protein) from CM-Sephadex was applied to a column of DEAE-Sephadex (40 × 3 cm), the unadsorbed material was washed out with 300 ml of 0·02M sodium phosphate buffer (pH 7·0) and elution was then continued with a linear NaCl gradient (0—0·5M-NaCl in the same buffer; the total volume 450 ml). Abscissa: volume of eluate in ml (V). Ordinate: absorbance at 280 nm (A_{280}) and substrate inactivation (in μmol/g); hatched area — enzymatic activity.

TABLE I
Yield of Individual Purification Steps

Purification step	Protein content mg/ml	Degree of purification
Liver homogenate	90	1
105000 *g* supernatent	23	3
Eluate from CM-Sephadex	30	4
Eluate from DEAE-Sephadex (NaCl gradient). Dialysis	0·72	72
Supernatant after 50% saturation by $(NH_4)_2SO_4$	0·34	88
Suspension of precipitate after 70% saturation by $(NH_4)_2SO_4$	3·8	132
Eluate from Sephadex G 100	1·5	150

buffer. The same buffer was used for washing out unadsorbed material from the column. These proteins represented approximately 60% of the total protein material applied to the column and were not enzymically active. The column was then eluted with a linear NaCl gradient in phosphate buffer. A typical elution diagram is shown in Fig. 1. The adsorbed inactivating enzyme was eluted from the column in the range of 0·25 – 0·3M-NaCl. This purification step resulted in 72fold purification of the inactivating enzyme.

The saturation of enzymically active eluate from DEAE-Sephadex to a 50% ammonium sulfate concentration removed some inactive protein material. The salt concentration in the soluble protein fraction was then increased to 70% and the precipitate, containing the inactivating enzyme, was dissolved in a small amount of $1 . 10^{-6}$M-EDTA. This preparation already had 132times higher specific activity than the liver homogenate. When the material was passed through a column of Sephadex G 100, the degree of purification increased; the specific activity reached 150times that of the liver homogenate. In some experiments, however, the preparation lost enzymic activity. The kinetic studies were therefore performed with the material precipitated at 70% ammonium sulfate saturation.

Enzyme Kinetics and Mechanism of Action

The enzyme preparation was incubated with various concentrations of the substrates DCOT-1, DCOT-6, DDCOT and oxytocin. The Lineweaver–Burk plot revealed that higher concentrations of oxytocin and the two mono-carba analogues, DCOT-1 and DCOT-6, caused inhibition of the reaction rate. This was not observed in the case of DDCOT. The K_m value for oxytocin, DCOT-1 and DDCOT was $2·5 – 3·3 . 10^{-6}$M and $6·25 . 10^{-6}$M for DCOT-6. Paper chromatography of the cleavage products of DDCOT showed the release of glycineamide from the C-terminus of the peptide.

DISCUSSION

The common characteristic of all the enzymes inactivating the neurohypophysical hormones oxytocin and vasopressin as yet known appears to be their exopeptidase mode of action. In the course of the last few years, it has been established that several tissues contain enzymes which split off the terminal glycineamide from the linear chain of neurohypophysial hormones[5,6]. Their specificity somewhat resembles that of trypsin and chymotrypsin. Together with the two-step inactivation mechanism found in the liver[14] which includes reduction followed by aminopeptidase cleavage, carboxyamidases might play an important role in regulating the concentration of oxytocin and vasopressin in tissues. After finding that the enzymic system inactivating deamino-carba analogues is localized in the subcellular fraction containing cytoplasmatic proteins[7], we applied a series of purification steps which resulted in an enzymic preparation with a specific activity 150 times higher than that of the crude liver homogenate.

The partially purified enzyme preparation obtained after precipitation by ammonium sulfate was used for studies of the kinetics of inactivation of oxytocin and three of its deamino-carba analogues. The Lineweaver–Burk plot revealed a difference between peptides containing at least on sulphur atom (oxytocin, DCOT-1 and DCOT-6) and the analogue without sulphur in its molecule (DDCOT). In the case of the first three substrates, inhibition by higher substrate concentrations was noticeable. When DDCOT was the substrate this phenomenon was not observed in the same concentration range. Substrate inhibition is explained by the existence of several binding sites on the enzyme; the binding of more substrate molecules to the enzyme can change the spatial arrangement of the complex in such a way that the rate of the catalytic reaction decreases[15]. The nonapeptides studied are compounds with a relatively complicated structure. The enzyme–substrate interaction might include several binding sites. At higher substrate concentrations several substrate molecules could be bound to the enzyme and the rate of the catalytic reaction might consequently be lower. In our experiments, substrate inhibition seems to be correlated with the presence of sulphur in the ring of the hormone molecule. Thus, not only the possibility of disulphide interaction (in the case of oxytocin), but the presence of a sulphur atom in the molecules of DCOT-1 and DCOT-6 (resulting in higher electrophility of the mono-carba analogues as compared with DDCOT which has no sulphur in its molecule) is sufficient to produce substrate inhibition. The slight difference between the affinities of DCOT-6 and the other substrates to the enzyme (manifested by the K_m values) cannot be adequately explained as yet.

As the rate of DDCOT inactivation was not inhibited at higher substrate concentrations, we used this substrate for studying the mechanism of peptide cleavage by the purified enzyme preparation. It was possible to prove the liberation of glycineamide from the substrate by means of paper chromatography.

The carboxyamidases isolated from kidneys[6] and uteri[5] were found to release glycineamide from the peptide chain; our results from experiments with the liver enzyme indicate a similar reaction mechanism. It is probable that the enzymic release

of glycineamide from oxytocin in tissues is one of the important factors which regulate the concentration of the hormone in the organism.

It is difficult to compare our purification procedure for the isolation of rat liver carboxyamidase with methods used for the isolation of carboxyamidases from other sources, namely the rat uterus[5] and kidney[6], as different purification steps were applied. However, some differences are obvious. Gel filtration on Sephadex G 150 of the partially purified kidney enzyme fraction showed that the inactivation enzyme was not a homogenous protein. The eluate yielded three peaks containing oxytocin-inactivating enzymes. The elution volume of the third peak indicates that the protein has a molecular weight lower than 10^5. The enzyme we isolated from the liver can also be assumed to have a lower molecular weight than 10^5; it appeared, however, to be more homogenous during gel filtration.

Like the liver enzyme, the kidney enzymes were sensitive to the presence of heavy metal cations, which is indicated by the finding that the enzymic activity decreased in the absence of EDTA.

We wish to express our thanks to Dr K. Jošt for kindly providing us with the oxytocin analogues.

REFERENCES

1. Walter R., Shlank H., Glass J. D., Schwartz I. L., Kerényi T. D.: Science *173*, 827 (1971).
2. Du Vigneaud V., Lawler H. C., Popenoe E. A.: J. Am. Chem. Soc. *75*, 4880 (1953).
3. Campbell J., Thysen B., Fun Sun Chu: Life Sci. *4*, 2129 (1965).
4. Barth T., Pliška V., Rychlík I.: This Journal *32*, 1058 (1967).
5. Glass J. D., Dubois B. M., Schwartz I. L., Walter R.: Endocrinology *87*, 730 (1970).
6. Koida M., Glass J. D., Schwartz I. L., Walter R.: Endocrinology *88*, 633 (1971).
7. Suska-Brzezińska E., Fruhaufová L., Barth T., Rychlík I., Jošt K., Šorm F.: This Journal *37*, 2289 (1972).
8. Tuppy H., Nesvadba H.: Monatsh. *88*, 977 (1957).
9. Bohus B., Ader R., de Wied D.: Hormones and Behaviour *3*, 191 (1972).
10. Jošt K.: This Journal *32*, 218 (1971).
11. Jošt K., Šorm F.: This Journal *36*, 234 (1971).
12. Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1951).
13. Munsick R. A.: Endocrinology *66*, 451 (1960).
14. Rychlík I.: *Oxytocin, Vasopressin and their Structural Analogues* (J. Rudinger, Ed.), p. 131. Pergamon Press, Oxford 1964.
15. Webb J. L.: *Enzyme and Metabolic Inhibitors*, Vol. 1, p. 111. Academic Press, New York, London 1963.

Translated by the author (L. F.).