CHYMOTRYPTIC CLEAVAGE OF DEAMINO ANALOGUES OF OXYTOCIN

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Like oxytocin, deamino analogues of oxytocin are inactivated by chymotrypsin. In oxytocin the bond between leucine and glycineamide is split. By contrast, in deamino-1-carba-oxytocin which belongs to the carba series, the bond between tyrosine and isoleucine is hydrolysed preferentially; the bond between leucine and glycineamide is split at a much slower rate. On the average, the $K_{m(app)}$ values for deamino analogues are lower by three orders of magnitude than those estimated for oxytocin.

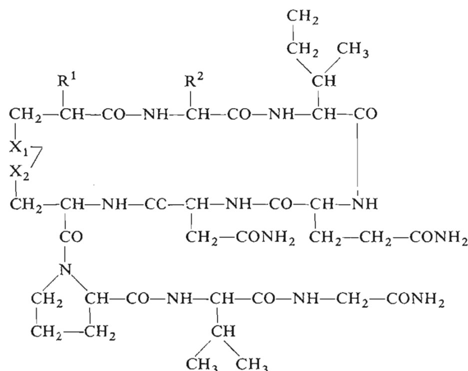
In model experiments the natural neurohypophysial hormone oxytocin is a substrate of chymotrypsin, which hydrolyses its peptidic bond between leucine in position 8 and terminal glycineamide[1,2]. Using a set of oxytocin molecule fragments we showed that hydrolysis occurred if the C-terminal peptidic fragment was sufficiently long, *i.e.* if it was composed of at least 4 amino acids[3]. With the increasing length of peptidic chain, the affinity to chymotrypsin as well as the hydrolytic rate also increased. We did not succeed in proving that the peptidic bond formed by tyrosine and isoleucine in oxytocin was hydrolysed. One reason for the stability of the mentioned bond was seen in the presence of the primary amino group in the vicinity of the bond concerned. We explained the stability of the peptides, leucylglycineamide and prolyl-leucyl-glycineamide towards chymotrypsin in a similar way.

The synthesis of deaminooxytocin and other derivatives made it possible to verify our original assumption. In this work we try to investigate how the absence of the primary amino group may influence the mode of chymotrypsin action and to study the potential effect of other structural modifications on the accessibility of the bonds concerned to chymotryptic cleavage.

EXPERIMENTAL

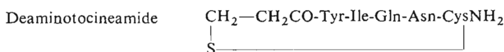
Materials

Oxytocin was purchased from Spofa, Prague, 1-deaminooxytocin[4], [6,1-cystathionine]oxytocin = = 1-carba-oxytocin[5], [6,1-β-deaminocystathionine]oxytocin = deamino-1-carba-oxytocin[6], [6,1-α-deaminocystathionine]oxytocin = deamino-6-carba oxytocin[8] [6,1-β-deaminocystathionine,



Compound	$X_1 - X_2$	R^1	R^2
Oxytocin	S—S	$—NH_2$	$—CH_2C_6H_4OH$-(*p*)
1-Carba-oxytocin	CH_2—S	$—NH_2$	$—CH_2C_6H_4OH$-(*p*)
Deaminooxytocin	S—S	—H	$—CH_2C_6H_4OH$-(*p*)
Deamino-1-carba-oxytocin	CH_2—S	—H	$—CH_2C_6H_4OH$-(*p*)
Deamino-6-carba-oxytocin	S—CH_2	—H	$—CH_2C_6H_4OH$-(*p*)
Deamino-dicarba-oxytocin	$CH_2—CH_2$	—H	$—CH_2C_6H_4OH$-(*p*)
[1,6-α-Aminopimelic acid]oxytocin	$—CH_2—$	—H	$—CH_2C_6H_4OH$-(*p*)
[1,6-Deamino-homolanthionine]oxytocin	—S—CH_2—S—	—H	$—CH_2C_6H_4OH$-(*p*)
Deamino-1-carba-[Ile[2]]oxytocin	CH_2—S	—H	$—CH(CH_3)—CH_2—CH_3$

Deaminotocineamide



2-isoleucine]oxytocin[7], [1,6-α-aminopimelic acid]oxytocin[9], [1,6-α-aminosuberic acid]oxytocin = = deamino-dicarba-oxytocin[8], [1,6-deaminohomolanthionine]oxytocin[10], 1-deaminooxytocin--1,6-hexapeptide amide = deaminotocinamide[11] were prepared at the Department of Organic Synthesis of our Institute and kindly offered by Drs K. Jošt and M. Zaoral.

Bovine chymotrypsin was purchased from Worthinghton, USA.

Methods

Biological assay. The biological activity of samples was determined by a test on the isolated rat uterine horn according to Holton[12] in the modification of Munsick[13]. The amount of substance remaining after the incubation with chymotrypsin was compared with the amount of substance incubated under the same conditions but in the absence of chymotrypsin.

Incubation conditions. Oxytocin or its analogues (final concentration 1—10 μM) were incubated in 50 mM sodium phosphate buffer, pH 7·5 with chymotrypsin (final concentration 1 μM) for 0—90 min. The enzymic reaction was stopped by boiling for 5 min. The remaining biological activity was determined by the uterotonic test[12,13] and expressed as percentage of the blank sample. The initial rate of hydrolysis was determined graphically. For the compounds deamino-oxytocin, deamino-1-carba-oxytocin, deamino-6-carba-oxytocin and deamino-dicarba-oxytocin, the values $K_{m(app)}$ and k_{cat} were determined according to Zerner and Bender[14]. The rate of inactivation of the other analogues was compared under standard conditions (concentration of analogue 5 μM, that of chymotrypsin 1 μM, time of incubation 60 min). The remaining amount of biologically active compounds was determined by the uterotonic test.

Determination of N-*terminal amino acids.* 2·5 mg of deamino-1-carba-oxytocin was incubated for 120 min with 1 mg of chymotrypsin in 20 mM sodium phosphate buffer, pH 7·5, total volume 2·5 ml. The enzymic reaction was stopped by boiling for 5 min and the reaction mixture was freeze-dried. In a portion of the sample, the N-terminal amino acids of splitting products were determined by the dansylation method[15]. Dansylated amino acids were separated by chromatography on polyamide sheets[16].

RESULTS AND DISCUSSION

Under the experimental conditions when the peptidic bond formed by leucine and glycineamide in oxytocin is slowly hydrolysed by chymotrypsin[2], deaminooxytocin and the majority of carba analogues are completely hydrolysed. In order to investigate the differences in the hydrolytic rates of the individual carba analogues of oxytocin, the experimental conditions were modified, *i.e.* the concentration of chymotrypsin was sharply reduced. The magnitude of inactivation of the individual analogues is presented in Table I. Neither oxytocin nor carba-1-oxytocin were inactivated under these conditions. In contrast with an analogue having an enlarged ring, the analogue of deaminooxytocin with a diminished ring was inactivated only slightly.

The analysis of the enzymic products of deamino-1-carbaoxytocin hydrolysis revealed the cleavage of the peptidic bond between tyrosine and isoleucine and only slight cleavage of the bond between leucine and glycineamide. The ratio between the rates of hydrolysis of these two bonds may be estimated by comparing the inactivation rates of deamino-1-carba-oxytocin, deamino-1-carba[Ile^2]oxytocin and deamino-tocinamide. The structural modifications of the latter analogues exclude one of the two possible sites of chymotrypsin attack (Fig. 1). On the basis of our experimental values one can estimate that the peptidic bond between leucine and glycineamide is hydrolysed at least 10 times more slowly than the bond between tyrosine and isoleucine. The rate of enzymic hydrolysis was determined by measuring the decrease

TABLE I

Chymotryptic Cleavage of Some Analogues of Oxytocin

Compound	Inactivation, %
Oxytocin	0
1-Carba-oxytocin	0
Deamino-carba-1-[Ile^2]oxytocin	10
[1,6-α-Aminopimelic acid]oxytocin	10
Deamino-6-carba-oxytocin	11
Deamino-dicarba-oxytocin	20
Deaminooxytocin	24·5
Deaminotocinamide	56
[1,6-Deamino-homolanthionine]oxytocin	84
Deamino-1-carba-oxytocin	92

of biological activity in the incubated samples. Therefore, we may assume that the kinetic parameters given in Table II include the rates of the cleavage of both bonds concerned. Furthermore, we assume that both splitting products are inactive in the uterotonic assay. It is evident from the Table II that the elimination of the primary amino group from the oxytocin molecule enhanced the affinity of the analogues to chymotrypsin on the average by three orders of magnitude. Although the elimina-

FIG. 1

Schematic Presentation of Possible Modes of Inactivation of Some Deamino Analogues of Oxytocin by Chymotrypsin

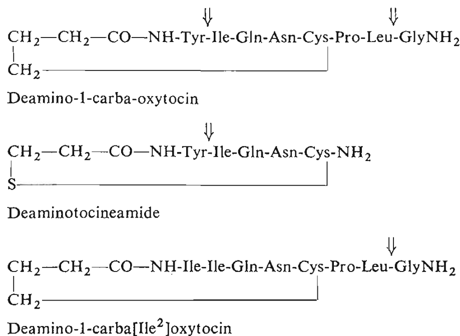


TABLE II

Hydrolysis of Oxytocin Analogues by Chymotrypsin

Compound	$K_{m(app)}$	k_{cat}	$k_{cat}/K_{m(app)}$
Oxytocin[a]	$1.8 \cdot 10^{-3}$	$2.02 \cdot 10^{-3}$	1·12
[Ala[1],Ala[6]]oxytocin[a]	$2.25 \cdot 10^{-3}$	$2.65 \cdot 10^{-2}$	11·8
[Cys(Me)[1],Cys(Me)[6]]oxytocin[a]	$1.43 \cdot 10^{-3}$	$2.25 \cdot 10^{-2}$	15·7
[Ser[1],Ser[6]]oxytocin[a]	$2.28 \cdot 10^{-3}$	$7.95 \cdot 10^{-3}$	3·5
[Ser(Bzl)[1],Ser[6]]oxytocin[a]	$6.2 \cdot 10^{-3}$	$3.2 \cdot 10^{-3}$	0·52
Deaminooxytocin[b]	$1.38 \cdot 10^{-6}$	$3.6 \cdot 10^{-2}$	26 000
Deamino-1-carba-oxytocin[b]	$5.5 \cdot 10^{-6}$	$2.85 \cdot 10^{-1}$	51 300
Deamino-6-carba-oxytocin[b]	$2.5 \cdot 10^{-6}$	$8 \cdot 10^{-2}$	32 000
Deamino-dicarba-oxytocin[b]	$6.25 \cdot 10^{-6}$	$9.6 \cdot 10^{-2}$	12 500

[a] Ref. 2; [b] this paper.

tion of the primary amino group from oxytocin results in complete resistance of these analogues to aminopeptidase splitting it also exposes a new peptidic bond to chymotrypsin.

In order to estimate whether the duration of the biological effect is in some way related to the metabolic stability of the analogues we compared the ratio of $K_{m(app)}$ and k_{cat} values (the ratio indicates the accessibility of the analogue to chymotrypsin splitting) of four deamino analogues presented in Table II with their indices of persistence of the uterotonic activity[17]. When we arranged the analogues according to their index of persistence: deamino-dicarba-oxytocin (the most protracted analogue) < deamino-6-carba-oxytocin < deaminooxytocin < deamino-1-carba-oxytocin we obtained a set similar to that of the analogues arranged according to their decreasing resistance to chymotrypsin: deamino-dicarba-oxytocin (the most stable analogue) < deaminooxytocin < deamino-6-carba-oxytocin < deamino-1-carba-oxytocin. Even though both series are rather similar, it would be premature to assume the chymotryptic mode of analogue inactivation in the rat uterus.

The elimination of the primary amino group endows the oxytocin analogues with lower resistance to chymotryptic attack. The other structural modifications realized in the hexapeptide cycle only modify the rate of hydrolysis of the analogues by chymotrypsin.

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