THE EFFECT OF NON-CODED AMINO ACIDS ON THE DEGRADATION OF OCYTOCIN ANALOGS WITH α-CHYMOTRYPSIN*

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Oxytocin and a series of its analogs, containing a non-coded amino acid in the carboxy-terminal linear part, were incubated with α-chymotrapsin and their degradation was monitored using high performance liquid chromatography (HPLC). Degradation of the analogs was markedly slower than that of oxytocin, its rate depending on the structure of the amino acid in position 8. Whereas in oxytocin the cleavage takes place at the leucine–glycine peptide bond in its carboxy--terminal moiety, no cleavage at this position was observed in the analogs; these were cleaved only at the tyrosine–isoleucine bond in the cyclic part. The same type of cleavage was observed in analog with a disulfide bond in the C-terminal oxytocin tripeptide. The degradation sites in oxytocin and its analogs were determined using the amino acid and sequentional analysis of the products isolated from the incubation mixture by preparative HPLC.

Enzymatic degradation of peptide hormones in living organisms leads to short duration of their effect, limiting thus their application in human as well as veterinary medicine. Therefore, there is great interest in preparation of analogs of biologically active peptides in which structural modifications should suppress the enzymatic cleavage of peptide bonds, with resulting prolonged activity. Also in the region of neurohypophyseal hormones oxytocin and vasopressin* a series of analogs of enhanced resistance toward enzymatic cleavage has been prepared: the modification consisted in replacing individual amino acid moieties by non-coded amino acids[3,4].

For oxytocin itself it has been found[5,6] that the presence of a primary N-terminal amino group in its molecule prevents α-chymotryptic cleavage of the tyrosine–isoleucine peptide bond. Accordingly, this bond is cleaved in deamino-oxytocin and its analogs[7]. However, the differences in the rates of this cleavage indicate that also conformational changes in the twenty-membered ring in the amino-terminal part

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** The nomenclature and symbols of amino acids and peptides obey the published recommendations[1,2]. In addition to the common symbols we use the following symbols: Pen penicillamine, Cle 1-aminocyclohexanecarboxylic acid (cycloleucine), Tle 3,3-dimethyl-2-aminobutanoic acid (tert-leucine), Neo 4,4-dimethyl-2-aminopentanoic acid (neopentylglycine).

of the hormone have a significant effect on the cleavage of the tyrosine–isoleucine bond[4,8]. The cleavage takes place even in the antiparallel dimer with forty-membered ring, [8-lysine]vasopressin, in which the primary amino group is preserved[9]. It was therefore of interest to study the effect of the amino acid moiety in position 8 on the sensitivity of the mentioned bond toward chymotrypsin.

The existing studies on chymotryptic cleavage of oxytocin and its analogs show that the enzyme–substrate ratio markedly affects the enzyme-catalyzed hydrolysis. Whereas with chymotrypsin in the ratio 1 : 300 no cleavage of oxytocin was observed, at a substantially higher ratio (1 : 3) the enzyme cleaved 10% of the C-terminal glycinamide after 3 hours and 100% after 24 hours of incubation[5]. A similar chymotrypsin-catalyzed hydrolysis of oxytocin was described by Barth and coworkers[10].

H-Aaa-Tyr-Ile-Gln-Asn-Cys-R

I, Aaa = Cys; R = Pro-Leu-Gly-NH_2
Ia, Aaa = Cys; R = Pro-Leu-OH
II, Aaa = Cys; R = Pro-Neo-Gly-NH_2
III, Aaa = Cys; R = Pro-Tle-Gly-NH_2
IV, Aaa = Cys; R = Pro-Cle-Gly-NH_2
V, Aaa = Cys; R = Pro-MeLeu-Gly-NH_2
VI, Aaa = Cys; R = Pro-Leu-$\Psi(CH_2S)$-Gly-NH_2
VII, Aaa = Cys; R = Cys-Leu-Cys-NH_2
VIII, Aaa = Pen; R = Pro-Cle-Gly-NH_2

H-Aaa-Tyr-OH H-Ile-Gln-Asn-Cys-R

Ib, Aaa = Cys; R = Pro-Leu-OH
Ic, Aaa = Cys; R = Pro-Leu-Gly-NH_2
IIa, Aaa = Cys; R = Pro-Neo-Gly-NH_2
IIIa, Aaa = Cys; R = Pro-Tle-Gly-NH_2
IVa, Aaa = Cys; R = Pro-Cle-Gly-NH_2
Va, Aaa = Cys; R = Pro-MeLeu-Gly-NH_2
VIa, Aaa = Cys; R = Pro-Leu-$\Psi(CH_2S)$-Gly-NH_2
VIIa, Aaa = Cys; R = Cys-Leu-Cys-NH_2
VIIIa, Aaa = Pen; R = Pro-Cle-Gly-NH_2

From the viewpoint of the anticipated higher resistance toward enzymatic cleavage, this work concerns first of all such oxytocin analogs in which the leucine moiety in position 8 is replaced by non-coded amino acids such as neopentylglycine (*II*) (ref.[12]), tert-leucine (*III*) (ref.[13,14]), cycloleucine (*IV*) (ref.[12]), N-methylleucine (*V*) (refs[15]), or in which the leucine–glycine peptide bond is replaced by the isosteric

thioether bond (*VI*) (refs[16]) (for a preliminary communication see ref.[11]). The studied series further includes [Cys^7, Cys^9]oxytocin (*VII*) (refs[17]) with preserved leucine moiety in position 8 and disulfide bridge between the cysteine residues in positions 7 and 9, and [Pen^1, Cle^8]oxytocin (*VIII*) (refs[18]), stabilized toward enzymatic cleavage also in the cyclic part of the molecule.

In the present study we used a high enzyme–substrate ratio (1·2 : 1, w/w) to get unequivocal information on the studied stabilizing effect of the non-coded structure in position 8 on the corresponding peptide bond. The degradation of oxytocin and its analogs was followed by high performance liquid chromatography (HPLC). Products of the chymotryptic cleavage were isolated by semipreparative HPLC; they were characterized by amino acid and sequentional analysis and mass spectrometry.

Differences in basicity of the degradation products relative to the original peptides *I* – *VIII* were determined by electrophoresis.

EXPERIMENTAL

Oxytocin was purchased from Léčiva, Prague (SPOFA, Czechoslovakia) and α-chymotrypsin (bovine pancreas, research grade, c. 45 U/mg) from SERVA (Feinbiochemica, Heidelberg, F.R.G.). The oxytocin analogs *II* – *VIII* were prepared, purified and characterized in our laboratory as well as in the laboratory of Professor V. J. Hruby, University of Arizona (Tucson, U.S.A.).

For HPLC an SP 8700 instrument with an SP 8400 UV detector and SP 4100 integrator (Spectra Physics, Santa Clara, U.S.A.) was used. The analytical HPLC was carried out on a 15 × 0·4 cm column packed with Separon SIX C-18, 7 μm (Laboratorní přístroje, Prague, Czechoslovakia), flow rate 42 ml/h, detection at 222 nm, mobile phase methanol with 0·05% aqueous trifluoroacetic acid. Values of k' were determined with a mobile phase containing 50% of methanol. Preparative HPLC was done on a 25 × 1·0 cm Vydac protein and peptide column C-18 (The Separations Group, Hesperia, U.S.A.), flow rate 180 ml/h, mobile phase gradient of 0–50% methanol in 0·05% aqueous trifluoroacetic acid.

The amino acid analyses were performed on a Durrum D-500 amino acid analyzer (Durrum Instrum. Corp., Palo Alto, U.S.A.), following hydrolysis in 6M-HCl for 20 h at 110°C. The sequentional analyses were carried out on a 470A Protein Sequencer (Applied Biosystems, Palo Alto, U.S.A.). The electrophoreses were done in a moist chamber on a Whatman 3MM paper (20 V/cm) in a pyri- dine–acetate buffer (pH 5·7) and in 6% acetic acid (pH 2·4); detection with ninhydrin. Mass spectroscopy with FAB technique was used for determination of M^+ of the corresponding peptides (VG ANALYTICAL, England).

Incubation of Oxytocin (*I*) and Analogs *II* – *VIII* with α-Chymotrypsin

The incubation experiments were carried out at 37°C with a mixture containing $5{\cdot}0 \cdot 10^{-1}$ mg/ml of the cleaved peptide and $6{\cdot}0 \cdot 10^{-1}$ mg/ml of α-chymotrypsin in 20 mM sodium phosphate buffer of pH 7·8. The remaining amount of non-cleaved peptide in the reaction mixture was determined at appropriate time intervals by means of HPLC (Table I). On the preparative scale, the main HPLC peaks resulting from the enzymatic degradation were collected, the solutions concentrated and lyophilized and the peptides analyzed. The characteristics of individual compounds are given in Table I. Incubation of oxytocin afforded three compounds; from all other analogs only one product was isolated.

TABLE I

Characteristics of the products of chymotryptic cleavage of oxytocin and analogs *II*—*VIII*

Compound	Formula M.w./M + H$^+$	$E^{Gly}_{2,4}$ $E^{His}_{5,7}$	k'[a] t[b], min	Amino acid analysis				Edman degradation		
				Asp Cys	Glu Ile	Pro Leu	Gly Tyr	1st step	2nd step	3rd step
Ia	$C_{41}H_{62}N_{10}O_{12}S_2$	0·47	3·1	1·0	1·08	1·03	—	[i]	Tyr	Ile
	951·1/951	0·00	31·5	2·0	0·95	1·05	0·91			Tyr
Ib	$C_{41}H_{64}N_{10}O_{13}S_2$	0·46	1·6	1·02	1·10	1·08	—	Ile	Gln	Asn
	969·1/969	0·00	26·3	1·88	1·0	1·07	0·92		Tyr	
Ic	$C_{43}H_{68}N_{12}O_{13}S_2$	0·62	1·4	1·09	1·10	1·03	1·02	Ile	Gln	Asn
	1 025·2/1 026	0·40	25·1	1·87	1·00	1·03	0·87		Tyr	
IIa	$C_{44}H_{70}N_{12}O_{13}S_2$	0·54	2·0	1·00	1·06	1·00	1·04	Ile	Gln	Asn
	1 039·3/1 040	0·32	26·2	1·84	0·94	0·95[c]	0·89		Tyr	
IIIa	$C_{43}H_{68}N_{12}O_{13}S_2$	0·67	1·4	1·05	1·09	1·04	1·08	Ile	Gln	Asn
	1 025·2/1 026	0·32	22·5	1·92	1·00	1·08[d]	0·92		Tyr	
IVa	$C_{43}H_{66}N_{12}O_{13}S_2$	0·62	1·9	1·07	1·08	0·96	1·01	Ile	Gln	Asn
	1 023·3/1 024	0·41	24·3	1·88	1·00	0·93[e]	0·84		Tyr	
Va	$C_{44}H_{70}N_{12}O_{13}S_2$	0·63	1·7	1·05	1·08	1·02	0·99	Ile	Gln	Asn
	1 039·3/1 040	0·41	25·2	1·90	1·00	—[f]	0·84		Tyr	
VIa	$C_{43}H_{69}N_{11}O_{12}S_3$	0·62	2·0	0·98	1·08	1·07	—	Ile	Gln	Asn
	1 028·3/1 029	0·31	28·1	1·86	1·00	—	0·97		Tyr	
VIIa	$C_{42}H_{66}N_{12}O_{13}S_4$	0·58	2·0[g]	1·04	1·08	—	—	Ile	Gln	Asn
	1 075·4/1 076	0·30	16·1	3·4	1·00	0·96	0·89		Tyr	
VIIIa	$C_{45}H_{70}N_{12}O_{13}S_2$	0·60	1·6	1·00	1·04	0·96	1·04	Ile	Gln	Asn
	1 051·5/1 052	0·38	23·8	1·02[h]	1·00	0·98	0·88		Tyr	

[a] 50% MeOH/0·05% trifluoroacetic acid; [b] retention time in preparative run (gradient 0—50% MeOH in 0·05% trifluoroacetic acid in 30 min); [c] Neo; [d] Tle; [e] Cle; [f] MeLeu not determined; [g] 25% MeOH/0·05% trifluoroacetic acid; [h] Pen 0·94; [i] traces of Cys[1] degradation.

RESULTS AND DISCUSSION

As shown by HPLC, incubation of oxytocin (*I*) with α-chymotrypsin under the given conditions (enzyme : substrate 1·2 : 1, w/w) led to complete degradation of the peptide within 5 h (Table II). The reaction gave products characterized by one HPLC peak of higher, and two peaks of lower capacity factor than oxytocin (Table III). Isolation and characterization of the products revealed that the higher capacity factor peak ($k' = 3{\cdot}1$) corresponded to des-9-glycinamide-oxytocin (*Ia*) and the two other peaks ($k' = 1{\cdot}4$ and $1{\cdot}6$) were due to derivatives of oxytocin and des-9-glycinamide-oxytocin, respectively, in which the tyrosine–isoleucine peptide bond in the cyclic moiety had been broken (*Ic* and *Ib*, respectively). After five-hours' incubation these products were isolated by gradient HPLC. Their population in the reaction mixture (52% of *Ib*, 26% of *Ic* and 22% of *Ia*) was rather surprising in the light of the described preferential cleavage of the glycinamide moiety from oxytocin molecule at lower α-chymotrypsin concentrations in the incubation mixture[5,10]. This result indicates that, under the conditions used, the cleavage of the leucine–glycine bond is accompanied by simultaneous cleavage of the tyrosine–isoleucine peptide bond, which leads to a relatively high content of compound *Ic* in the final reaction mixture. It is also probable that the prevailing leucine–glycine bond cleavage, observed at the beginning of the incubation, is accompanied by the subsequent tyrosine–isoleucine bond hydrolysis in des-9-glycinamide-oxytocin (*Ia*) under formation of compound *Ib* which entirely predominates after five-hours' incubation.

The chymotryptic degradation, observed with oxytocin, is reflected also in degradation of the studied analogs *II* – *VIII*; in these cases, however, its course is simpler.

TABLE II

Degradation of oxytocin (*I*) and its analogs (*II* – *VIII*) with α-chymotrypsin. Content of intact peptide in % is given

Peptide	Time in min								
	20	40	60	80	100	120	140	160	180
Oxytocin (*I*)	40	25	21	12	10	7	5	4	3
[Neo^8]OXT (*II*)	72	56	46	35	30	24	22	20	18
[Tle^8]OXT (*III*)	78	70	67	60	57	54	53	50	48
[Cle^8]OXT (*IV*)	83	75	70	66	62	58	57	56	55
[MeLeu^8]OXT (*V*)	98	88	85	79	78	77	76	75	75
[$^8\Psi^9$,CH_2S]OXT (*VI*)	68	60	53	48	42	35	32	28	25
[Cys^7,Cys^9]OXT (*VII*)	78	70	65	60	56	53	51	50	48
[Pen^1,Cle^8]OXT (*VIII*)	90	80	75	70	66	64	63	62	61

Most of the analogs (monitored by HPLC similarly as in the case of oxytocin) were degraded much more slowly than oxytocin (Table II), the reaction rates for the individual compounds differing markedly from each other (e.g. only 25% of [8-N-methylleucineoxytocin] (*V*) was hydrolyzed after 3 h). All the studied analogs were cleaved under formation of only one product characterized by a strong HPLC peak representing more than 95% of the products. The k' values of all these new peaks were invariably lower than those of the original analogs (Table III). This characteristic feature has been found also with the analog *VI* which in the position 8–9 of the backbone contains the thioether, resistant to α-chymotryptic cleavage. Also the analog *VII*, with the disulfide bond in the oxytocin carboxy-terminal tripeptide, exhibited an enhanced resistance to chymotrypsin and, similarly, the degradation product was characterized by an HPLC peak of k' lower than that of the starting analog. The amino acid composition of the chymotryptic cleavage products (*IIa* to *VIIIa*), isolated by semipreparative HPLC, was identical with that of the analogs *II* – *VIII*. Both the sequentional analysis and electrophoresis have proven that in these peptides the peptide bond cleavage takes place only in the cyclic moiety at the carboxyl side of the tyrosine residue and that during the incubation with α-chymotrypsin no amino acid is cleaved off. Similarly as in the two enzymatic cleavage products of oxytocin, *Ib* and *Ic*, Edmann degradation has found isoleucine as the first amino acid. The electrophoretical mobility of the mentioned cleavage products was identical with that of the corresponding analogs (as found also for the mobility

TABLE III

Capacity factors k' and retention times of oxytocin (*I*), analogs *II* – *VIII* (A) and those of the main products of α-chymotryptic degradation *Ia* – *Ic*, *IIa* – *VIIIa*

Peptide	k'[a]				t, min[b]			
	A	Products			A	Products		
Oxytocin (*I*)	2·3	1·4	1·6	3·1	28·1	25·1	26·3	31·5
[NeO[8]]OXT (*II*)	3·1	2·0			29·2	26·9		
[Tle[8]]OXT (*III*)	1·8	1·4			26·4	22·5		
[Cle[8]]OXT (*IV*)	2·3	1·9			27·2	24·3		
[MeLeu[8]]OXT (*V*)	2·2	1·7			28·9	25·2		
[$^{8}\Psi^{9}$,CH_2S]OXT (*VI*)	3·2	2·0			31·7	28·1		
[Cys[7],Cys[9]]OXT (*VII*)	4·3[c]	2·0[c]			21·0	16·1		
[Pen[1],Cle[8]]OXT (*VIII*)	2·0	1·6			26·5	23·8		

[a] 50% Methanol in 0·05% trifluoroacetic acid; [b] retention time in min in the gradient preparation using 0–50% methanol in 0·05 trifluoroacetic acid; [c] 25% methanol in 0·05% trifluoroacetic acid.

of product *Ic* arising from oxytocin by tyrosine–isoleucine cleavage), contrary to the mobility of the neutral products *Ia* and *Ib*, formed by loss of glycinamide in the cleavage of the leucine–glycine bond.

It is evident that even at extremely high enzyme–substrate ratio the peptide bond between the amino acid in position 8 and glycinamide in the studied oxytocin analogs is not cleaved. The replacement of the leucine moiety by a non-coded amino acid worsens probably the spatial fit for the interaction between this part of molecule and the active site of the enzyme which for this reason cannot catalyze the removal of glycinamide. Therefore the stabilization of the leucine–glycine peptide bond leads to an overall slower cleavage of these analogs as compared with oxytocin. This manifests itself also in the case of the analog *VII* with retained leucine moiety in position 8, in which the required interaction of its side-chain with α-chymotrypsin (and thus cleavage) is apparently hindered by the disulfide structure of cystinamide in positions 7 and 9.

On the other hand, it has been proven that in analogs *II* – *VIII* the tyrosine–isoleucine peptide bond in the cyclic moiety (for α-chymotrypsin not easily accessible) is cleaved, similarly as in oxytocin. Whereas the cleavage of the Tyr-Ile bond in analogs *II* and *VI* proceeds by approximately the same rate as in oxytocin, the analogs *III*, *IV* and *VII* were cleaved more slowly and for the analog *V* (containing N-methylleucine) it was difficult to follow quantitatively the cleavage at all. Also the analog *VIII*, which contains a penicillamine moiety in position 1 in addition to substitution in position 8 (cycloleucine), exhibits a markedly enhanced stability of this peptide bond. Evidently, the proven chymotryptic cleavage of the Tyr-Ile bond in the studied peptides is influenced by the structure of the amino acid moiety in position 8. The stability of this bond increases with increasing steric hindrance in the proximity of the peptide backbone in position 8, as if particularly the MeLeu, Cle and Tle moieties fixed (under the chosen experimental conditions) such conformation of the carboxy--terminal tripeptide in the oxytocin analogs that cancels the spatial arrangement of the cyclic moiety (particularly the tyrosine side-chain), suitable for chymotryptic hydrolysis of the Tyr-Ile bond. The observed dependence may also be explained assuming that the interaction of tyrosine with α-chymotrypsin and the subsequent, though slow, cleavage of the Tyr-Ile bond is enabled just by the flexibility of the carboxy-terminal tripeptide in oxytocin which increases the probability of a conformation (or a set of conformations) suitable for interaction with the active site of the enzyme. The restricted flexibility of the tripeptide chain due to a bulky amino acid in position 8 decreases this probability.

Conformational considerations concerning the interaction between the cyclic and linear moieties in the molecule of oxytocin and its analogs are to a certain extent supported by the CD spectra. The restricted flexibility in position 8 results in well--discernible spectral changes. Particularly affected is the intensity of the positive composite CD-band at 223 – 230 nm, resulting from superposition of an aromatic

B_{1u} transition band in the tyrosine side-chain at about 228 nm and an amide $n-\pi^*$ transition band at shorter wavelengths[19]. Both these components depend obviously on conformation of the structural unit represented by the tyrosine side-chain and the neighbouring amide groups (cf. e.g. refs[18,19]). The intensity of the composite band in a neutral phosphate buffer pH 7·5 (i.e. in the medium we have used for the incubation of oxytocin and its analogs with α-chymotrypsin) is depicted in Fig. 1. In the spectrum of oxytocin and analogs *III*, *IV* and *V* the aromatic component is the predominant contributor to the composite band, the bands in spectra of Cle--containing analogs are also considerably contributed by the amide band. The negative elipticity of compound *V* is the result of superposition of a positive band on a negative background.

As follows from Fig. 1, the analogs in which the Tyr-Ile peptide bond is hydrolyzed considerably more slowly (Table II) exhibit positive composite CD bands whose intensities are significantly different from that of the corresponding band of oxytocin. The largest absolute deviations were observed with analogs *IV* and *VIII*, containing a Cle moiety, and the analog *V* with the MeLeu moiety in position 8, which are the most resistant analogs concerning the Tyr-Ile bond cleavage. However, as indicated by the CD spectra, in the case of these two structural modifications, the character of the conformational change in the tyrosine region si probably different.

The mentioned observations on peptide bond cleavage in oxytocin (*I*) and its analogs *II* – *VIII* lead to the conclusion that the generally higher stabiliy of these analogs is due to the absolute resistance of their C-terminal peptide bond to α-chymotrypsin. The cleavage of the bond on the carboxyl side of tyrosine in position

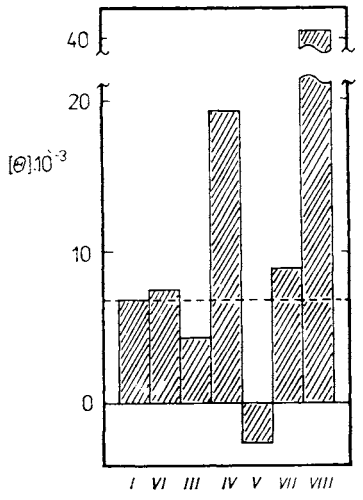

FIG. 1

Intensity of the positive composite band at 223–230 nm in the CD spectra of oxytocin (*I*), [$^{8}\Psi^{9}$, CH_2S]OXT (*VI*), [Tle^{8}]OXT (*III*), [Cle^{8}]OXT (*IV*), [MeLeu^{8}]OXT (*V*), [Cys^{7}, Cys^{9}]OXT (*VII*) and [Pen^{1}, Cle^{8}]OXT (*VIII*), in 0·05M phosphate buffer of pH 7·5

in analogs with relatively flexible arrangement in position 8 (i.e. [Neo^8]OXT (*II*), [$^8\psi^9$,CH_2S]OXT (*VI*)) is comparable with the same cleavage in oxytocin and des-9--glycinamide-oxytocin. In analogs *III* and *VII* the presence of Tle in position 8 or bridging the leucine with a disulfide bond, which reduces the flexibility of this part, increase the resistance of the Tyr-Ile peptide bond to α-chymotrypsin. In the CD spectrum of analogs *III* and *VII* this substitution manifests itself only by a slight change in intensity of the composite positive band in the case of analog *VII* we observed also marked changes of short-wavelength bands in the region 195 to 200 nm, contributed by other aromatic and amide transitions[17].

Only the presence of cycloleucine (*IV*) (the effect is further enhanced by introducing penicillamine into position 1 (*VIII*)) or N-methylleucine (*V*) in position 8 increases significantly the stability of the Tyr-Ile peptide bond and at the same time significantly influences the CD spectra in the region sensitive to conformation of the tyrosine residue in position 2 (Fig. 1).

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