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Primary Structure of Somatostatin, A Hypothalamic Peptide That Inhibits the Secretion of Pituitary Growth Hormone

(somatotropin release/Edman degradation/dansylation/mass spectrometry)

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ABSTRACT **Somatostatin, a peptide isolated from ovine hypothalamic tissue that inhibits the release of radioimmunoassayable growth hormone *in vitro* from rat or human pituitary cells or *in vivo* in rats, has the primary structure H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-OH. The structure was established by submitting the carboxymethylated peptide, the carboxymethylated tryptic digest, and the chymotryptic digest of the peptide to Edman degradation. Degradation products were analyzed by amino-acid analysis, as well as in some cases by determination of N-termini by dansylation or by determination of phenylthiohydantoins by mass spectrometry.**

We have recently reported the isolation from (sheep) hypothalamic extracts of a peptide that specifically inhibits at $\geqslant$ 1 nM the *in vitro* secretion of immunoreactive rat or human growth hormones, and that is similarly active *in vivo* in rats (1). This peptide will be referred to as somatostatin (see ref. 1). We report here details of the determination of the primary sequence of this peptide.

MATERIALS AND METHODS

The starting material was a side fraction obtained from the purification of extracts of sheep hypothalamic fragments for the characterization of the releasing factors of the gonadotropins, in a program similar to that previously described for luteinizing hormone-releasing factor (LRF) (2). Details of the isolation procedure will be published elsewhere (Brazeau *et al.*, in preparation). In summary, an ethanol–chloroform–acetic acid–water 810:100:5:90 extract of about 500,000 sheep hypothalamic fragments (2 kg) was partitioned in the system 0.1% acetic acid–*n*-butyl alcohol–pyridine 11:5:3, and the material from the organic phase was then partitioned in *n*-butyl alcohol–acetic acid–water 4:1:5. The aqueous phase from the second distribution was subjected to ion-exchange chromatography on carboxymethyl-cellulose, whereupon a basic fraction with somatostatin activity was well separated from the luteinizing hormone-releasing factor zone. The biologically active fraction was further purified by gel filtration on "Sephadex G-25" in 0.5 M acetic acid (R_F of somatostatin = 0.5), and finally by liquid partition chromatography (3) on a "Sephadex G-25" support in *n*-butyl alcohol–acetic acid–water 4:1:5 (R_F = 0.4). The final product, 8.5 mg, contained 75% amino acids by weight, and showed only traces of peptide impurities upon thin-layer chromatography and electrophoresis.

Papain, leucine aminopeptidase, α-chymotrypsin (3-times crystallized), and crystalline trypsin (treated with L-[tosylamido-2-phenyl]ethyl chloromethyl ketone, as in ref. 4) were obtained from Worthington. Reagents were reagent or analytical grade.

Amino-Acid Analysis. Peptides were hydrolyzed in 200 μl of 6N HCl–0.5% thioglycollic acid (5) or 50 μl of 3N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole (6). Tube contents were degassed by freezing and thawing at 50–100 μm Hg pressure, and the sealed tubes were incubated 15–20 hr at 110°. The hydrolyzates from *p*-toluenesulfonic acid hydrolysis were partially neutralized with 50 μl of 2N NaOH for amino-acid analysis. HCl hydrolyzates were concentrated to dryness under reduced pressure and dissolved in buffer for analysis.

Total enzymic hydrolysis (7) of the peptide (20 nmol) was performed by digestion with papain (1 μg) in 100 μl of 0.05 M NH_4OAc (pH 5.3)–0.02 M 2-mercaptoethanol buffer for 2 hr at 37° followed by, after addition of 2–3 drops of glacial acetic acid, a second incubation of the lyophilized residue with leucine aminopeptidase (1.5 μg) in 100 μl of 0.02 M Tris-acetate–1 mM $MnCl_2$–5 mM $MgCl_2$ (pH 8.5) buffer for 15 hr at 37°. The second incubation mixture was acidified with 2–3 drops of acetic acid, lyophilized, and dissolved in 0.2 N Na citrate buffer (pH 2.2) for amino-acid analysis.

Analyses were performed with a Beckman/Spinco model 119 amino-acid analyzer equipped with a Beckman/Spinco model 138 automatic sample injector. Single column methodology (Beckman/Spinco technical bulletin ATB-087) was used with a 56 × 0.9-cm column of Beckman AA-15 resin at 55°. Buffers were prepared as described in Beckman/Spinco bulletin A-TB-020G with two modifications: the first buffer of the three-buffer system was adjusted to pH 3.26 rather than 3.49 and 5% (v/v) *n*-propyl alcohol was added to the third buffer (pH 6.41) to obtain more rapid elution of Trp. At a flow rate of 70 ml/hr, buffer changes were set at 110 min for the switch from pH 3.26 (0.2 M Na ion) to 4.14 (0.2 M Na ion) buffer and at 172 min for the switch from pH 4.14 to 6.41 (1.6 M Na ion) buffer.

Determination of Free Sulfhydryl Groups. Quantitative analysis of free SH groups with 5,5′-dithiobis-(2-nitrobenzoic acid), Ellman's reagent, (8), was as described by Habeeb (9).

Abbreviations: Cys(Cm), *S*-carboxymethylcysteine; dansyl, dimethylaminonaphthalene-5-sulfonyl-, PITC, phenylisothiocyanate; Pth, phenylthiohydantoin; Ptc, phenylthiocarbamyl-.

Carboxymethylation. Reduction of the intact or trypsin-digested somatostatin with 2-mercaptoethanol and conversion of the SH groups generated to *S*-carboxymethyl (Cm) derivatives with iodoacetate (10) were performed in volatile buffers. To 1.8 mg (0.74 μmol) of peptide was added 30 μl (0.43 mmol) of 2-mercaptoethanol in 200 μl of 0.5 M triethylammonium acetate buffer (pH 8.5). The mixture was incubated for 4.5 hr at room temperature, after which 10 μl of glacial acetic acid was added and the solution was lyophilized.

To the reduced peptide in 400 μl of 0.5 M pyridine acetate–5 mM 2-mercaptoethanol buffer (pH 6.0) was added 100 μl of 0.1 M iodoacetic acid in the same buffer. After incubation at room temperature for 20 min with occasional mixing, 6 μl (100 μmol) of 2-mercaptoethanol was added and the mixture was allowed to stand at room temperature for an additional 15 min; 20 μl of acetic acid was then added and the solution was lyophilized. The residue was twice dissolved in 200 μl of 0.1 M acetic acid containing 6 μl of 2-mercaptoethanol and lyophilized overnight.

Reduction and alkylation of 180 nmol of peptide digested with trypsin was performed similarly.

Edman Degradation. Sequential degradation of peptides based on the method of Edman was performed essentially as described (11) with a few modifications: coupling with phenylisothiocyanate (PITC) was done with 5% dimethylallylamine in 50% pyridine, adjusted to pH 9.5 with trifluoroacetic acid. Because of difficulties due to the hydrophobic nature of the peptide, it was necessary to reduce the extent of the butylacetate extraction procedure in these experiments. After the first Edman degradation cycle (coupling and cleavage) of 1.8 mg of carboxymethylated somatostatin, the partially degraded peptide was suspended in 150 μl of 0.5 M pyridine acetate buffer (pH 6.5) and extracted with 0.5 ml of *n*-butylacetate (containing 50 μl/liter of dithioethane). After centrifugation, most or all of the peptide was found suspended in the aqueous phase, insoluble in both phases. The precipitate was combined with 150 μl of buffer wash of the butylacetate phase and lyophilized. The residue was dissolved in 50% pyridine–5% dimethylallylamine buffer, in which it was soluble, for the next Edman cycle. This procedure was repeated until the 5th Edman cycle, whereupon the residual degraded peptide became soluble in butylacetate. An equal volume of cyclohexane was added to the butylacetate phase to precipitate the peptide, which was combined with the residue to the aqueous phase for the next cycle. In the 6th Edman cycle, the buffer suspension of peptide (150 μl) was extracted with 0.5 ml of 1:1 (v/v) cyclohexane–butylacetate. After the 7th Edman cycle, the peptide partitioned completely into the butylacetate phase (Table 1) when equal volumes of buffer and butylacetate were shaken together; the 8th Edman degradation was, therefore, performed on the butylacetate phase. After the 9th Edman cycle, the peptide again started to partition into the aqueous phase, so this and subsequent degradation cycles were performed by the extraction procedure described for the 1st cycle; thus, 150 μl of buffer was extracted with 0.5 ml of butylacetate, and the butylacetate was washed with 150 μl of buffer.

The mixtures of peptides in the tryptic or chymotryptic digests were sequenced according to the method described for the first cycle above. Contents of each of the butylacetate phases and each of the aqueous phases from the carboxymethylated tryptic digest were sequenced. Because some of the difficulties in sequencing the intact peptides may have been due to incomplete coupling of PITC with the peptide (see *Results* and *Discussion*), 5 μl of neat PITC was added after the usual 1 hr of coupling and incubation was continued for an additional hour during the sequencing of the carboxymethylated trypsin digest.

Aliquots were taken for amino-acid analysis, as well as for analysis by the dansyl end-group method with ^{14}C-labeled dansyl derivatives (11), and for direct determination of Pth-derivatives by mass spectrometry as described below. Control samples of each enzyme were carried through the sequencing procedures.

Mass Spectrometry. Silylated Pth-derivatives were determined by gas chromatography–mass spectrometry as described (11). Some of the 2-anilino-thiazolinone derivatives were analyzed by mass spectrometry with a direct inlet system. The thiazolinone derivatives need not be converted to the corresponding thiohydantoins because they undergo thermal rearrangement to the thiohydantoin from the heat of the probe during mass spectrometric analysis (12). The temperature of the probe was slowly increased to boil off the excess PITC until the molecular ion of the Pth-derivative was observed.

Digestion with Chymotrypsin. A 150-nmol aliquot of somatostatin was incubated 19 hr at 37° in 80 μl of 0.1 M NH_4OAc buffer (pH 8.1) with 7.5 μg of α-chymotrypsin.

Digestion with Trypsin. A 450-nmol aliquot of somatostatin was incubated for 15 hr at 37° in 200 μl of 0.1 M NH_4OAc–1 mM $CaCl_2$ buffer (pH 8.1) with 40 μg of trypsin. An aliquot containing 270 nmol of peptide was subjected to acetylation and permethylation for direct mass spectrometry (15). The remaining aliquot (180 nmol) was subjected to sequencing by Edman degradation as described above.

Determination of C Termini. C-Terminal amino acids were determined by hydrazinolysis and dansylation (11).

RESULTS AND DISCUSSION

Analysis of amino acids obtained from somatostatin after acid hydrolysis in 6 N HCl–0.5% thioglycollic acid gave the molar ratios Ala (0.9), Gly (1.1), Cys (0.2), Cys Cys (1.0), Lys (2.0), Asp (1.0), Phe (3.3), Trp (0.5), Thr (2.0), Ser (0.8), and NH_3 (1.1). Enzymic hydrolysis gave the ratios Ala (0.9), Gly (0.9), Lys (2.0), Phe (3.4), and Trp (0.9); Asn, Thr, and Ser were not well resolved, giving a total of about 3.6 mol/mol of peptide; residual NH_4OAc interfered with calculation of NH_3; no cysteine (retention time = 78 min) or cystine (retention time = 117 min) was recovered, but a peak with a retention time of 86 min was tentatively identified as a

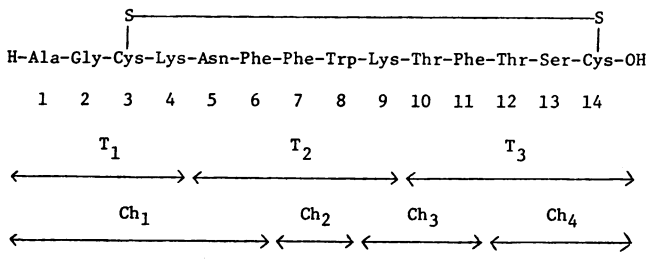

FIG. 1. Peptide fragments observed after treatment of ovine somatostatin with trypsin (T_1–T_3) or chymotrypsin (Ch_1–Ch_4).

TABLE 1. *Amino-acid analysis of Edman degradation products of carboxymethylated somatostatin*

Edman cycle†	Amino-acid molar ratios*							
	Ala	Gly	Cys(Cm)	Lys	Asx	Phe	Thr	Ser
0	0.9	1.0	1.2‡	1.8	1.0	3.2	1.9	0.8
1A	—	0.9	1.5	1.5	1.0	2.7	1.8	0.8
2A	—	0.1	1.3	1.3	1.0	2.6	1.6	0.9
3A	—	0.1	0.7	1.4	1.0	2.6	1.6	0.9
4A	—	0.1	0.8	0.8	1.0	2.5	1.7	0.9
5A	—	0.2	1.0	0.9	0.5	2.5	2.1	0.9
6A	—	0.1	0.9	0.9	0.4	2.2§	2.0	0.9
7B	—	—	0.8	0.8	0.3	1.7§	2.0	0.9
8A	—	0.1	0.8	0.7	0.1	0.9§	2.0	1.1
8B	—	0.1	0.7	1.0	0.3	1.6§	2.0	1.0
9A	—	0.1	0.7	0.7	0.2	1.2§	2.0	0.8
9B	—	0.2	0.6	0.9	0.4	1.1§	2.0	0.9
10A	—	0.3	1.2	0.1	0.1	0.6§	2.0	1.1
10B	—	—	0.8	0.6	0.4	1.7§	2.0	0.9
11A	—	0.1	1.0	0.1	0.2	0.4	1.1	0.7
12A	—	0.1	—	—	—	—	1.0	0.7
13A	—	—	0.9	—	—	—	1.0§	0.9
14A	—	—	1.0	—	—	—	0.4	0.6
15A¶	—	—	1.0	—	—	—	—	—

* Numbers underlined indicate the amino acids subtracted in each cycle.

† *A* signifies that the analyses of Edman degradation products were performed on the aqueous phase, *B* on the butylacetate phase.

‡ Ratio of Cys(Cm) to Cys = 1.2.

§ Difficulties in these stages were due to failures in the coupling (or cleavage) reactions of the Edman degradation procedure, as well as to the solubility of the degraded peptide in the organic phase during the extraction of Pth-derivatives in cycles 6–9. The 8th Edman cycle was performed on the products in the butylacetate fraction, 7*B*, and the 9th cycle on fraction 8*B*; the other sequencing reactions were done on the water-soluble fractions (see text).

¶ Unhydrolyzed.

mixed disulfide arising from cysteine and mercaptoethanol (13) in the buffer used in the enzymic hydrolysis. Using the color value for cystine, 2 mol of total cysteine residues per mol of peptide were calculated, but no conclusion could be drawn about the state of oxidation of the sulfur in the analytical samples because it was altered by the hydrolysis reagents. However, no free SH groups could be detected in somatostatin with Ellman's reagent, demonstrating that the peptide as isolated is in a disulfide form. Only one N-terminal amino acid, Ala, could be detected by dansylation or by sequencing (see below). The possibility that the peptide is a dimer or higher molecular weight polymer appears to be excluded by its behavior upon gel filtration. Therefore, somatostatin as isolated is probably a monomeric peptide with a disulfide bridge. The state of oxidation of the sulfur in somatostatin as it occurs in the *native* state, however, remains unresolved (1).

Sequencing of the carboxymethylated peptide by analysis by the subtractive method (Table 1) did not lead to an unequivocal sequence, because Trp was not recovered in the

TABLE 2. *Edman degradation of water-soluble materials in a carboxymethylated tryptic digest of somatostatin*

Edman cycle	Amino-acid molar ratios*									Dansyl amino acids found	Pth-Derivatives found†
	Ala	Gly	Cys(Cm)	Lys	Asx	Phe	Trp	Thr	Ser		
0	0.9	1.0	1.0	1.5	1.0	2.4	0.2	1.9	0.8	Ala,Asp,Thr	—
1	—	1.0	1.2	0.8§	0.2	0.9§	—§	1.0	0.8	Gly,Phe	Ala,Asn,Thr
2	—	0.1	1.2	0.6	0.2	—	—	1.0	0.8	Cys(Cm),Thr	Gly,Phe
3	0.1	0.2	0.7	0.8	0.2	—	—	0.2	0.8	—	Cys(Cm),Thr
4 Hydrolyzed	—	0.1	0.7	0.1	0.1	—	—	0.1	0.3	—	Lys,Ser
4 Unhydrolyzed	—	—	‡	—	—	—	—	—	—	—	—

* Numbers underlined indicate the amino acids subtracted in each cycle.

† In butylacetate phase.

‡ Found 7 nmol of Cys(Cm) of the theoretical 10 nmol. No Cys(Cm)–NH_2 was found.

§ At cycle 1 a peptide containing 2 Phe, 1 Lys, and an unidentified residue (assumed to be Trp) was extracted into the butylacetate phase (see Table 3 and text).

TABLE 3. *Edman degradation of butylacetate phases of carboxymethylated tryptic digest of somatostatin*

Degradation cycle*	Amino-acid molar ratios†									Dansyl amino acids found
	Ala	Gly	Cys(Cm)	Lys	Asp	Phe	Trp	Thr	Ser	
1	—	0.2	—	0.8	0.1	1.5	—	0.1	0.1	Phe
2	—	—	—	0.8	—	1.0	—	—	—	Phe
3	—	—	—	0.8	—	0.5	—	—	—	—
4	—	—	—	—	—	—	—	—	—	(ε-Ptc)Lys

* Analyses of cycles 1–3 were performed on aliquots of butylacetate phases; at cycle 4 the amino-acid analysis was not performed. Dansylation at cycle 4 was performed on the unhydrolyzed aqueous phase.

† Numbers underlined indicate the amino acids subtracted in each cycle.

analyses and difficulties caused by failures during cycles 6–9 and 13 in the coupling (or cleavage) reaction resulted in a net loss of two Edman cycles. Nevertheless, the data in Table 1, together with the known composition of somatostatin, support the conclusion that Edman reaction and subtraction of 2 Phe, 1 Lys, and possibly 1 Trp took place in cycles 5–10. Therefore, the partial sequences: H-Ala-Gly-Cys-Lys-Asn-(Phe, Phe, Trp, Lys)-Thr-Phe-(Thr, Ser)-Cys-OH or Ala-Gly-Cys-Lys-Asn-Phe-(Phe, Lys)-Thr-Phe-(Trp, Thr, Ser)-Cys-OH (with Trp being subtracted in the 13–15 cycle) can be proposed. Dansyl end-group analysis confirmed the N-terminal sequence Ala-Gly-Cys-Lys-Asx-Phe-(Phe, X)-Lys- (X denotes that no amino acid was identified), and mass spectrometry revealed Pth-derivatives of Ala, Gly, Cys, Lys, Asn, and Phe in cycles 1–6, respectively. Beyond the eighth cycle, evaluation by the dansyl end-group procedure became impossible because of the low recovery of peptide, and because in the aliquots required, residual reagents interfered with the analysis. An unidentified oil residue that was not extractable by butylacetate was particularly troublesome. This product may result from the use of dimethylallylamine buffer in silylated glassware (14). Mass spectrometric analysis also was difficult because of the inefficient extraction of thiazolinone under these conditions and because of the small amount of peptide remaining in the last cycles. The appearance of Cys-(Cm) in the unhydrolyzed sample from the last Edman cycle while no Cys(Cm)-NH_2 (retention time = 167 min) was found confirms that the C-terminus is the free acid.

After trypsin digestion of somatostatin followed by carboxymethylation, dansylation showed Ala, Asx, and Thr as N-termini; hydrazinolysis-dansylation of the products yielded Lys and Cys(Cm) as C-termini. Sequencing by Edman degradation of the carboxymethylated trypsin digest as followed by subtractive amino-acid analysis, dansylation, and mass spectrometry gave the results shown in Tables 2 and 3.

With the foregoing data and that of Tables 2 and 3, we can conclude that trypsin digestion resulted in three peptide fragments. The amino-terminal sequence previously obtained from the results of sequencing the intact carboxymethylated peptide (see above) has been established as H-Ala-Gly-Cys-Lys-Asn-Phe-; hence, the fragment T_1, Ala-Gly-Cys-Lys-(Fig. 1) would be expected from a tryptic cleavage of the N-terminus. This result accounts for the subtraction of Ala, Gly, Cys(Cm), and Lys in Edman cycles 1–4 of the water-soluble materials of the trypsin digest (Table 2). Another fragment was extracted into the butylacetate phase after the first Edman degradation, accounting for the loss of 2 Phe, 1 Lys, and possibly 1 Trp from the aqueous phase (Tables 2 and 3). The third peptide fragment (Table 2) could, therefore, be identified in the aqueous phase as having the sequence Thr-Phe-Thr-Ser-Cys-OH (T_3, Fig. 1). The appearance of free Cys(Cm) rather than Cys(Cm)-NH_2 in the unhydrolyzed sample of the 4th Edman cycle confirms that the C-terminus is a free acid. The results of dansylation analysis and direct determination of Pth-derivatives (Table 2) are also compatible with structures assigned to peptides T_1 and T_3. The data in

TABLE 4. *Edman degradation of chymotryptic digest of somatostatin*

Edman cycle	Amino-acid molar ratios*									Dansyl amino acids found	Pth-Derivatives found
	Ala	Gly	Cys†	Lys	Asp	Phe	Trp	Thr	Ser		
0	1.0	1.1	1.9	1.9	1.0	2.8	0.4	2.0	0.9	Ala,Phe,Trp	Phe§,Trp§
1	—	0.8	0.8	0.3	0.8	2.0	—	1.5	1.2	Lys,Thr Gly,Trp,Thr, Ser	Phe-Trp§ Ala,Thr
2	—	0.2	0.6	0.4	0.7	2.0	—	0.1	0.2	Phe‡	Trp,Thr,Ser
3	—	—	—	0.5	1.0	0.8	—	0.1	0.2	(ε-Ptc)Lys	—
4	—	—	—	—	1.0	1.0	—	—	—	Asp	—
5	—	—	—	—	0.4	1.0	—	—	—	Phe	—
6	—	—	—	—	—	—	—	—	—	—	—

* Numbers underlined indicate the amino acids subtracted in each cycle.

† Determined as cystine. Recoveries of cystine are usually variable during Edman degradation.

‡ Dansyl derivative of cystine is not detectable in this system.

§ Identified as underivatized Phe, Trp, and H-Phe-Trp-OH.

Table 3 show that a tetrapeptide, -Phe-Phe-X-Lys- was extracted by butylacetate after the 1st Edman cycle, since C-terminal Lys as dansyl-(ε-Ptc)Lys was found in the unhydrolyzed aliquot after the 4th Edman cycle. A failure in the coupling (or cleavage) reaction in the 3rd cycle was ruled out since the subtractive method shows a removal of Phe in that cycle and since no dansyl-Phe was found (high Phe values in the analyses after the 2nd and 3rd cycles probably result from the hydrolysis of Pth-Phe; this derivative would also be blocked to the dansyl reaction). Since all the other amino acids present in the analysis of the intact peptide have been accounted for, it can be presumed that the unidentified amino-acid residue in the third position was Trp, and that the peptide fragment T_2 (Fig. 1) is -Asn-Phe-Phe-Trp-Lys-, thereby establishing the primary sequence of somatostatin as that shown in Fig. 1. In confirmation of the location of the Trp, mass spectrometric analysis of the acetylated permethylated peptide from the trypsin digest showed the presence of a fragment with the sequence -Asn-Phe-Phe-Trp-Lys- (15).

The results of sequencing the products of chymotrypsin digestion of somatostatin (Table 4) are also compatible with the sequence shown in Fig. 1, chymotryptic cleavages yielding primarily the fragments Ch_1, Ch_2, Ch_3, and Ch_4, with smaller amounts of free Phe and Trp.

The linear tetradecapeptide H-Ala-Gly-Cys(SH)-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys(SH)-OH was synthesized by solid-phase methodology and converted to the oxidized form (Fig. 1) (Rivier *et al.*, in preparation). Both forms have the qualitative and quantitative biological activity of isolated somatostatin (1). The biological results obtained with the reduced form thus leave open the question of the reduced or oxidized state of the Cys residues in the peptide as it is recognized by the pituitary receptors; both the isolation procedure and the conditions used in the bioassays might convert one form into the other.

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