Isolation and Sequence Analysis of a Somatostatin-like Polypeptide from Ovine Hypothalamus†

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ABSTRACT: A large somatostatin-like polypeptide of apparent molecular weight 3000–4500 [4K somatostatin (SS)] was isolated from ovine hypothalamus. The polypeptide was obtained in the methionine sulfoxide form. Two microsequence analyses of 0.6 and 1.8 nmol of 4K SS were performed with a modified 890 C spinning cup sequencer. The sequencing data together with results of amino acid analysis and C-terminal end-group determination indicated that 4K SS was identical with somatostatin-28 (SS-28) isolated from procine upper small intestine and sequenced by Pradayrol et al. [Pradayrol, L., Jörnvall, H., Mutt, V., & Ribet, A. (1980) *FEBS Lett. 109*, 55–58]. No free cysteine sulfhydryl group could be detected, so that it was assumed that the two cysteine residues of ovine SS-28 formed an intramolecular disulfide bond. Besides the structure of SS-28, the N-terminal first 30 residues of an unknown polypeptide from ovine hypothalamus were sequenced as follows: H-Ile-Pro-Ile-Tyr-Glu-Lys-Lys-Tyr-Gly-Gln-Val-Pro-Met-Cys-Asp-Ala-Gly-Glu-Gln-Cys-Ala-Val-Arg-Lys-Gly-Ala-Arg-Ile-Gly-Lys-. Trypsin cleaved the somatostatin (SS) entity less selectively from ovine hypothalamic SS-28 than from rat hypothalamic 12000-dalton SS-like polypeptide (12K SS). Native ovine hypothalamic SS-28 was found to be highly potent to inhibit growth hormone release from cultured rat anterior pituitary cells. The results raised doubts that ovine SS-28 would be an SS precursor and indicated that SS-28 itself may possess regulatory functions.

We have previously demonstrated (Spiess & Vale, 1978, 1980) that acidic aqueous extracts of rat hypothalamus contained three different somatostatin-like polypeptides which were described as 12K, 4K, and 2K SS on the basis of their apparent molecular weights. These polypeptides were recognized by somatostatin (SS)[1] radioimmunoassays (RIA's) by using the centrally directed antibody S-201.

The two larger polypeptides, 12K and 4K SS, exhibited in the N-terminally directed SS RIA S-39 less than 5% of the activity found in RIA S-201, whereas 2K SS was equally active in both assays. On the basis of the specificity of the antibodies S-201 and S-39 (Vale et al., 1976, 1978), it was concluded that 12K SS and 4K SS were N-terminal modifications or extensions of SS or a small SS-like polypeptide. Mild tryptic digestion of 12K SS generated activity in RIA S-39, cleaving selectively a small SS-like species of SS size active in RIA's S-201 and S-39. This result was interpreted (Spiess & Vale, 1980) as an indication that 12K SS represented an N-terminal extension rather than an N-terminal modification of SS or an SS-like structure. In view of the selective tryptic cleavage, characteristic for precursors (Steiner et al., 1971; Goltzman et al., 1976), 12K SS was considered as a possible SS precursor.

Thus far, our knowledge about hypothalamic 4K SS is still limited. To provide more information about this polypeptide, we have isolated and characterized the ovine hypothalamic 4K SS which behaved in RIA's S-201 and S-39 as well as in gel filtration experiments like rat hypothalamic 4K SS (Vale et al., 1976; Spiess et al., 1979a). Ovine hypothalamic 4K SS had earlier been partially purified together with the growth hormone releasing fragment of myelin basic protein (Villarreal et al., 1976) and the bombesin-like peptide (Villarreal & Brown, 1978). The data of the sequence analysis of 4K SS (without details) have been published earlier (Spiess et al., 1980a,b).

Experimental Procedures

Peptides. SS and [Gly[15]]SS were provided by Dr. J. Rivier, The Salk Institute. Somatostatin-28 (SS-28) was a gift of Dr. N. Ling, The Salk Institute.

Isolation of Ovine Hypothalamic 4K SS. The starting material of this investigation originated from a side fraction produced during the purification of ovine gonadotropin releasing hormone carried out in the Laboratories for Neuroendocrinology of The Salk Institute (Burgus et al., 1976). Fractions of 4K SS extracted from 490000 ovine hypothalami were initially purified by ion-exchange chromatography, gel filtration (Villarreal et al., 1976), and partition chromatography (Villarreal & Brown, 1978).

(*1*) *Cation-Exchange Chromatography.* Lyophilized fractions of SSLI (10000–25000 hypothalamic fragments) obtained by partition chromatography were incubated for 15 min (23 °C) in 0.5 mL of 4 M urea (Schwarz/Mann, Orangeburg, NY) and 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) adjusted to pH 7.4 by aqueous NaOH. The mixture was diluted by 2 mL of 1 M urea and 0.05 M Hepes (pH 7.4) and 1.5 mL of H_2O and then applied to a CM Bio-Gel A (Bio-Rad) column (0.9 × 19 cm) equilibrated with 1 M urea and 0.05 M Hepes (pH 7.4). The flow rate was 6 mL/h. The column was eluted with a buffer mixture which was produced by mixing 100 mL of 1 M urea and 0.05 M Hepes (pH 7.4) and 100 mL of 1 M urea, 0.05 M Hepes, and 0.2 M NaCl (pH 8.0) to form a linear gradient of increasing ionic strength and pH. Fractions of SSLI (usually in the specific conductance range 4.7–6.8 $m\Omega^{-1}/cm$) were combined, concentrated by lyophilization, and desalted by gel filtration

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[1] Abbreviations used: DME medium, Dulbecco modified Eagle's medium; CM, carboxymethyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; RIA, radioimmunoassay; SEM, standard error of the mean; SS, somatostatin; SS-28, somatostatin-28; SSLI, somatostatin-like immunoactivity; TEAF, triethylammonium formate; TEAP, triethylammonium phosphate; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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through a Bio-Gel P-2 column (1.5 × 30 cm) equilibrated with 3 M acetic acid.

(*2*) *Reverse-Phase High-Pressure Liquid Chromatography (LC) before S-Carboxymethylation.* Fractions of SSLI of the cation-exchange chromatography (desalted and lyophilized) were dissolved in 3 M acetic acid and subjected to reverse-phase high-pressure LC by using a Hewlett-Packard 1084B liquid chromatograph equipped with an auto sampler, a variable wavelength detector, and a cryogenic valve allowing for cooling the column compartment by CO_2 gas. The chromatography was performed with Du Pont Zorbax CN columns (0.46 × 25.0 cm) equilibrated with mixtures of buffers A and B. Buffer A consisted of 0.08 M phosphoric acid, adjusted to pH 3.0 by triethylamine (TEAP system). Buffer B was composed of buffer A (40% v/v) and acetonitrile (60% v/v). The flow rate and column temperature were set to 1.0 mL/min and 23 °C, respectively. Usually, flat linear gradients such as from 25% to 34% buffer B within 30 min were used to elute ovine 4K SS. Approximately 0.5–2.5 mg of protein was processed in one run.

(*3*) *S-Carboxymethylation.* Fractions exhibiting SSLI eluted from Zorbax CN columns were combined and desalted by reverse-phase high-pressure LC by using the Zorbax CN columns described above. Desalting was accomplished by using steep linear gradients, employing a buffer system similar to the TEAP system mentioned above. The only difference was that 0.08 M phosphoric acid was replaced by 0.25 M formic acid (TEAF system). More details about TEAP and TEAF systems are described elsewhere (Rivier, 1978).

S-Carboxymethylation was performed as described earlier (Spiess et al., 1979b). The method represents a modification of the procedure of Crestfield et al. (1963). Ovine 4K SS (2 μg-equiv according to RIA S-201) purified and desalted by reverse-phase high-pressure LC was subsequently dried by lyophilization. Reducing buffer (150 μL) containing 8 M urea, 0.58 M Tris-HCl, 6.0 mM sodium EDTA, and 57.5 mM dithiothreitol (pH 8.7) was saturated with argon and added to the dry residue. The mixture was incubated at 50 °C for 30 min under argon. After the mixture was cooled to 23 °C, 25 μL of 0.66 M sodium iodoacetate in reducing buffer without dithiotreitol was added. The mixture was kept at 23 °C for 7 min, acidified by glacial acetic acid (final concentration 20% v/v), and applied to reverse-phase high-pressure LC using the Zorbax CN columns and the TEAF buffer system described above. This carboxymethylation procedure was also employed for synthetic peptides (5–500 μg of SS, SS-28, or [Gly^{15}]SS).

Amino Acid Analysis. Peptides (0.2–0.5 nmol) were hydrolyzed with 25 (or 50) μL of methanesulfonic acid and 0.2% tryptamine (Moore, 1972) containing norleucine as internal standard (110 °C, 24 h, or 140 °C, 3 h). After hydrolysis, 25 (or 50) μL of 3.5 N NaOH or LiOH was added. An aliquot of this mixture with 120–500 pmol/amino acid was subjected to amino acid analysis performed with a Beckman 121 MB amino acid analyzer which was equipped with a model 126 data system. Beckman AA-10 resin was packed in the sodium form (0.28 × 20 cm) or the lithium form (0.28 × 25 cm). Sodium or lithium citrate standard programs (Beckman) with ninhydrin detection (single column systems) were used. Peptides containing methionine sulfoxide were hydrolyzed by 25 μL of 4 M LiOH (110 °C, 24 h). The mixture was acidified by 29 μL of 4 M methanesulfonic acid and analyzed by the lithium system, resolving methionine sulfoxide from the other frequently occurring amino acids.

Sequence Analysis. Sequence analysis based on the Edman degradation of peptides was performed with a Beckman 890C spinning cup sequencer modified according to Wittmann-Liebold (Wittmann-Liebold, 1973; Wittmann-Liebold et al., 1976). This modification included the introduction of a conversion flask, allowing for automatic conversion of the 2-anilino-5-thiazolinone derivatives of the cleaved amino acids into the corresponding 3-phenyl-2-thiohydantoin derivatives (PTH-amino acids). The Beckman delivery valves were replaced by diaphragm valves pneumatically actuated to deliver reagents or solvents or to apply argon to the cup or converting flask. The original vacuum pumps were replaced by Alcatel 2030C vacuum pumps and protected by traps cooled in a Dewar flask which was automatically filled by liquid nitrogen. The vacuum block, delivery system, and converting flask were manufactured by Horst Graffunder and Heinz Kohls of the Max-Planck-Institut für Molekulare Genetik, Berlin.

The following reagents and solvents were used: 5% phenyl isothiocyanate in *n*-heptane (R1), 0.33 M Quadrol trifluoroacetic acid (pH 8.9) (R2), heptafluorobutyric acid (R3), 25% (v/v) trifluoroacetic acid (R4), benzene (S1), 0.1% (v/v) acetic acid in ethyl acetate (S2), 0.3 mM dithioerythritol in *n*-butyl chloride (S3), 25% (v/v) acetonitrile in methanol (S4). All reagents were purchased from Beckman, with the exception of trifluoroacetic acid which was obtained from Pierce. The solvents were purchased from Burdick and Jackson. All reagents and solvents except Quadrol were redistilled.

The sequence program was developed on the basis of the programs earlier designed for the modified sequencer (Wittmann-Liebold et al., 1976; Hunkapiller & Hood, 1978). The program contained one coupling step (42 °C, 29 min) and a single cleavage (42 °C, 300 s). Conversion was performed at 52 °C for 55 min.

Usually, 6 mg of poly(*N*,*N*,*N*′,*N*′-tetramethyl-*N*-trimethylenehexamethylenediammonium diacetate) (Polybrene) was used as a peptide carrier in the cup (Tarr et al., 1978). Polybrene was extensively purified by adsorption and ion-exchange chromatography prior to introduction into the cup as an aqueous solution. Approximately 8–10 sequencing runs were performed before the peptide was added. More details of the sequencing program and the Polybrene purification will be described elsewhere (J. Spiess, unpublished experiments).

Identification of PTH-amino Acids. PTH-amino acids were identified and quantitated by reverse-phase high-pressure LC by using the liquid chromatograph described previously and Du Pont Zorbax ODS columns (0.46 × 25 cm). Usually, one-third of the fraction of each sequencer cycle was applied to one high-pressure LC run.

All common PTH-amino acids were resolved within 25 min by using an acetonitrile–TEAP buffer system at pH 5.7 or 5.9. Serine was identified as a PTH-Ser product eluting before PTH-Ala as described by Hunkapiller & Hood (1978). The calibration factor of this product was determined with radioactively labeled serine subjected to one sequencing cycle. Threonine was determined as PTH-dehydrothreonine. Details of the reverse-phase high-pressure LC of PTH-amino acids will be described elsewhere (J. Spiess and J. Heil, unpublished experiments).

The modified sequencer and the reverse-phase high-pressure LC method of PTH-amino acid identification allowed direct sequence analysis of 25–30 residues of 0.6–1.5 nmol of peptide applied to the cup. The repetitive yield was usually higher than 95%.

Radioimmunoassays. Two SS radioimmunoassays (RIA's) were employed: the N-terminally directed RIA S-39 (Vale et al., 1976) and the centrally directed RIA S-201 (Vale et al., 1978).

Table I: Purification of 4K SS from 49 380 Ovine Hypothalami

purification step	fraction	SSLI[a] (nmol of SS)	protein[b] (μg)	sp act. (pmol of SS/ μg of protein)	yield[c] (%)
	partition chromatography[d]	16.1	20590	0.8	100
1	cation-exchange chromatography	10.4	6292	1.7	65
2	reverse-phase high-pressure LC before carboxymethylation	3.6	143	25.2	22
3	reverse-phase high-pressure LC after carboxymethylation	0.22	9	24.4	

[a] SSLI was determined by RIA S-201. [b] Protein was determined by amino acid analysis after hydrolysis (24 h, 110 °C) with 4 M methanesulfonic acid and 0.2% tryptamine. [c] The yield was expressed as percent of the SSLI obtained by partition chromatography. [d] Details of the partition chromatography are described elsewhere (Villarreal & Brown, 1978).

Bioassay. SS-like biological activity was assayed by the inhibition of growth hormone secretion from rat anterior pituitary cells (Vale et al., 1975). Primary cell cultures were placed in plastic dishes (approximately 5×10^5 cells/dish) and maintained for 4 days in Hepes-modified Dulbecco modified Eagle's (DME) medium containing 10% fetal calf serum. Following a repeated change of medium, the cells were incubated with peptide in 1.5 mL of Hepes-modified DME medium containing 2% fetal calf serum and 0.4 mM 3-isobutyl-1-methylxanthine (IBMX) (4 h, 37 °C). The medium was removed and assayed for growth hormone by NIAMDD rat pituitary hormone distribution program kits prepared by A. Parlow.

Affinity Chromatography with Concanavalin A. Binding of ovine 4K SS to concanavalin A was investigated by a procedure previously applied to large forms of ACTH (Eipper et al., 1976). This procedure was slightly modified by using a different Con A buffer: 0.02 M sodium phosphate, 0.7 mM $MgCl_2$, 1 mg/mL bovine serum albumin (Pentex), 1.0 M NaCl, and 0.1% (v/v) Triton X-100, pH 7.0. Thyroid-stimulating hormone (a glycoprotein) iodinated by $Na^{131}I$ could be bound by these columns.

Trypsin Digestion. Ovine 4K SS partially purified by cation-exchange chromatography was incubated at 37 °C in 0.1 M ammonium acetate and 10 mM $CaCl_2$ (pH 7.0) with TPCK-trypsin (Worthington). The final concentrations of 4K SS and TPCK-trypsin were initially 23–30 nM SS equivalents (RIA S-201) and 1.7–16.7 ng/mL, respectively. The protein weight ratio of enzyme to substrate varied from 1:700 to 1:7000. After defined time intervals, aliquots were drawn, acidified by glacial acetic acid (final concentration 20%, v/v), and diluted by RIA buffer for analysis in RIA's S-201 and S-39.

Results

The specific activity of ovine hypothalamic 4K SS after initial purification by gel filtration, ion-exchange (Villarreal et al., 1976), and partition chromatography (Villarreal & Brown, 1978) was approximately 0.8 pmol of SS equivalents (RIA S-201) per microgram of protein. With the assumption of similar affinities of 4K SS and SS to antibody S-201, a homogeneous fraction of 4K SS would be expected to have a specific activity of approximately 320 pmol of SS equivalents per microgram of protein. The degree of purity of the initial fractions was accordingly estimated to be in the range 0.3–1%.

These fractions were subjected to ion-exchange chromatography, desalted by gel filtration, and further purified by reverse-phase high-pressure LC by using Zorbax CN columns and triethylammonium phosphate buffer. After these purification steps (steps 1 and 2, Table I), 4K SS reached a specific activity of 25–100 pmol of SS equivalents (RIA S-201) per microgram of protein, corresponding to a degree of purity in the range of 8–30%. Subsequently, the material was desalted by reverse-phase high-pressure LC, carboxymethylated by iodoacetate, and purified by reverse-phase high-pressure LC (step 3, Table I). At least four major components were separated by reverse-phase high-pressure LC of the carboxymethylated material (Figure 1). Carboxymethylated 4K SS (4K CM-SS) was identified by its SSLI (RIA S-201), which was only observed in the fraction eluted at 10.9 min (Figure 1). The specific activity of this 4K CM-SS fraction did not deviate significantly from the specific activity of the 4K SS fraction before the carboxymethylation (step 2, Table I). However, since carboxymethylated SS (CM-SS) exhibited only approximately 14% of the SSLI in RIA S-201 found for SS, it would not be surprising to observe a similar decrease in the affinity of 4K SS to antibody S-201 after carboxymethylation. Accordingly, the specific activity of 4K CM-SS could not be considered a valid indicator of the degree of purity of the 4K CM-SS fraction.

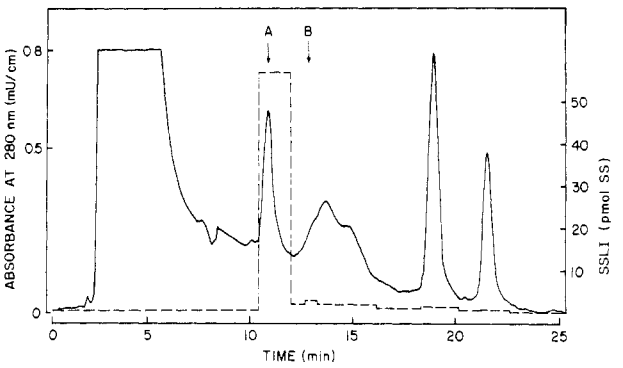

FIGURE 1: Reverse-phase high-pressure LC of ovine hypothalamic 4K SS (1.0 nmol of SS equivalents in RIA S-201, 41 μg of protein) after S-carboxymethylation. The Zorbax CN column was eluted with the acetonitrile–TEAF buffer system (pH 3.0). A linear gradient from 17.4% to 24% acetonitrile was employed. Absorbance (—) and SSLI in RIA S-201 (---) are plotted. (A) CM[Met-O^8]SS-28 (synthetic); (B) CM-SS-28 (synthetic).

The purity of 4K CM-SS could be estimated on the basis of amino acid analyses of the components obtained by reverse-phase high-pressure LC of the carboxymethylated material (Figure 1). Amino acid analysis was performed after hydrolysis with 4 M methanesulfonic acid in the presence of 0.2% tryptamine. The total amount of protein eluted from the Zorbax CN column (Figure 1) represented 79% of the protein which was subjected to desalting, carboxymethylation, and purification by reverse-phase high-pressure LC (step 3 of the purification, Table I). Approximately 96% of the total protein in the eluate was found in the four fractions eluted at 10.9, 13.6, 18.8, and 21.5 min (Figure 1). These fractions contributed 9, 24, 38, and 26%, respectively, to the total recovered protein. Amino acid analysis indicated that only the hydrolysate of the 4K CM-SS fraction (retention time 10.9 min, Figure 1) contained tryptophan. That the distribution of tryptophan and SSLI coincided was in agreement with the

Table II: Amino Acid Composition of Ovine Hypothalamic 4K SS

amino acid	acid hydrolysis[a] (mol of amino acid/ 3 mol of Lys)	sequence analysis	SS-28[b]
Asn		3	3
Asx	3.0		
Thr	1.8	2	2
Ser	3.0	3	3
Glu		1	1
Glx	1.3		
Pro	1.7	2	2
Gly	2.1	1	1
Ala	4.1	4	4
Cys	1.9[c]	2[c]	2
Val	0.0	0	0
Met	1.2	1	1
Ile	0.1	0	0
Leu	0.2	0	0
Tyr	0.1	0	0
Phe	2.7	3	3
Lys	3.0	3	3
His	0.0	0	0
Trp	0.7	1	1
Arg	2.1	2	2

[a] Peptide (0.3 nmol) was hydrolyzed for 24 h at 110 °C with 4 M methanesulfonic acid and 0.2% tryptamine. The values represent the averages of two analyzer runs. [b] From Pradayrol et al. (1980). [c] Cysteine was determined as carboxymethylcysteine.

finding that the presence of tryptophan in SS-like polypeptides is essential for their biological and immunological SS-like activity (Vale et al., 1976, 1978). Carboxymethylcysteine was only detected in the hydrolysates of the fractions eluted at 10.9, 18.8, and 21.5 min.

The analysis of the reverse-phase high-pressure LC (Figure 1) indicated that 4K SS was purified approximately 10-fold as 4K CM-SS under these highly resolving chromatographic conditions. In view of the purity of the 4K SS fraction before carboxymethylation, it was expected that the 4K CM-SS fraction would have a degree of purity of more than 80%. This expectation was in agreement with the amino acid composition data of the 4K CM-SS fraction (Table II). The experimental amino acid concentrations normalized to the lysine concentration deviated from integer values by an average of 10% (Table II). The degree of purity of the 4K CM-SS fraction (step 3 of the purification) was accordingly estimated to be at least 90%.

The amino acid composition of 4K CM-SS (Table II) was closely related to SS-28, isolated from porcine upper small intestine by Pradayrol et al. (1980). The only significant difference was the excess of glycine, amounting to one extra glycine residue in 4K SS compared to SS-28 (Table II). The relatively high glycine content of the 4K CM-SS fraction could not be explained by glycine or peptide contamination of solutions used for hydrolysis or amino acid analysis because no glycine was detected in control experiments analyzing these solutions. It was also unlikely that the 4K CM-SS fraction was contaminated by free glycine because it was found that free glycine was eluted significantly earlier than 4K CM-SS under the conditions of the reverse-phase high-pressure LC designed for the purification of 4K CM-SS.

The first sequence analysis of 4K SS was performed with a 4K SS fraction obtained from purification step 2 (Table I). The degree of purity of this fraction was approximately 10% on the basis of the specific activity (SSLI/protein weight). An aliquot of the fraction was desalted by gel filtration through a Bio-Gel P-2 column with 3 M acetic acid as eluant. Only

Table III: Sequence Analysis of the Major Component of the 4K SS Fraction Purified by Reverse-Phase High-Pressure LC[a]

cycle	PTH-amino acid	yield[b] (nmol)
1	Ile	2.36
2	Pro	0.96
3	Ile	1.52
4	Tyr	1.32
5	Glu	1.23
6	Lys	1.25
7	Lys	1.76
8	Tyr	1.18
9	Gly	0.97
10	Gln	0.75
11	Val	1.13
12	Pro	0.62
13	Met	0.68
14	X	
15	Asp	0.19
16	Ala	0.73
17	Gly	0.49
18	Glu	0.38
19	Gln	0.26
20	X	
21	Ala	0.62
22	Val	0.34
23	Arg	0.28
24	Lys	0.38
25	Gly	0.41
26	Ala	0.36
27	Arg	0.34
28	Ile	0.32
29	Gly	0.36
30	Lys	0.30

[a] The fraction applied to the cup (18.4 μg of protein according to amino acid analysis) had not been S-carboxymethylated. [b] The total yield per cycle was determined by reverse-phase high-pressure LC.

56% of the protein applied to this column was recovered in the eluate. The gel-filtered protein fraction was subjected to Edman degradation by a modified spinning cup sequencer. Two main components were degraded simultaneously. They represented approximately 80 and 15%, respectively, of the sequenced protein, based on the PTH-amino acid yields of the first cycles. For convenience, we will refer to the minor and major components sequenced here as component I and component II, respectively.

The amino acid sequence of component I from residue 1 to residue 26 did not deviate from the primary structure of porcine duodenal SS-28. Residues 17 and 24 could not be identified. It was concluded that component I represented a polypeptide closely related to SS-28. On the basis of comparable sequence analyses of synthetic SS-28, the amount of component I at the start of the sequence analysis was estimated to be approximately 0.6 nmol.

The first 30 residues of component II (Table III) were identified as H-Ile-Pro-Ile-Tyr-Glu-Lys-Lys-Tyr-Gly-Gln-Val-Pro-Met-X-Asp-Ala-Gly-Glu-Gln-X-Ala-Val-Arg-Lys-Gly-Ala-Arg-Ile-Gly-Lys.

It was expected that component II was identical with one of the side products of the purification of 4K CM-SS (Figure 1). Sequence analysis of the fraction eluted from the CN column at 18.8 min (Figure 1) indicated that this fraction contained only one N-terminally nonblocked polypeptide. The sequencing data of this carboxymethylated polypeptide confirmed the amino acid sequence of residues 1–30 of component II. Residues 14 and 20 were identified as cysteine residues. It was assumed that this polypeptide represented component II. Sequence analysis of the polypeptide eluted at 21.5 min (Figure 1) revealed that at least the 12 first residues of this

Table IV: Sequence Analysis of S-Carboxymethylated Ovine Hypothalamic 4K SS[a]

cycle	PTH-amino acid	yield[b] (pmol)
1	Ser	171[c]
2	Ala	947
3	Asn	495
4	Ser	134[c]
5	Asn	479
6	Pro	466
7	Ala	654
8	Met	625
9	Ala	588
10[d]	Pro	286
11	Arg	262
12	Glu	224
13	Arg	264
14	Lys	293
15	Ala	351
16	Gly	258
17	CM-Cys	222
18	Lys	197
19	Asn	235
20	Phe	281
21	Phe	333
22	Trp	215
23	Lys	196
24	Thr	24[e]
25	Phe	104
26	Thr	23[e]
27	Ser	23[c]
28	CM-Cys	58

[a] Carboxymethylated 4K SS (1.8 nmol) was applied to the sequencer cup. [b] The total yield per cycle was determined by reverse-phase high-pressure LC. [c] Serine residues were determined as PTH-serine derivatives (see Experimental Procedures). [d] For cycle 10, a second cleavage was introduced. [e] Threonine was determined as PTH-dehydrothreonine.

polypeptide also aligned with the N-terminal amino acid sequence of component II. On the basis of this finding, it could not be excluded that the sequence assigned to component II belonged to two closely related polypeptides which were present in the sequenced 4K SS fraction.

The primary structure of component II was compared with protein sequences listed in the Dayhoff file. A search program developed by R. Burgus and S. Minick was used. Component II showed mainly homologies to different cytochromes, hemoglobins, and myoglobins. Maximal alignment comprising five amino acids in a row was established with cytochrome *c* from rust fungus (Bitar et al., 1972) and gene J protein of bacteriophage PHI-X 174 (Sanger et al., 1977; Freymeyer et al., 1977). Residues 26–30 of component II aligned with residues 1, 2, 4, and 5 of hypothalamic tetradecapeptide (Schlesinger et al., 1978).

Sequence analysis was repeated with 1.8 nmol of 4K CM-SS from step 3 of the purification (Table I). Residues 1–28 could unambiguously be identified and quantitated (Table IV). The relative PTH-amino acid yields of two consecutive cycles were in agreement with the expected values obtained by sequence analysis of synthetic CM-SS-28 and other synthetic peptides. Sequencing of 4K CM-SS did not reveal any contamination by other peptides. Additional C-terminal amino acids could not be detected.

The sequencing data did not account for all amino acids found by amino acid analysis (Table II). Since it was possible that the extra glycine detected by the composition studies could represent the C terminus of 4K CM-SS, C-terminal end-group determination by using tritium label (Matsuo & Narita, 1975) was performed. Tritiated amino acids were identified and quantitated by amino acid analysis and scintillation counting of the hydrolyzed sample. With ~1.2 μg of peptide (purification step 3) applied to this method, it was found that 74% (2200 cpm) of the radioactivity incorporated into amino acids appeared in carboxymethylcysteine, indicating that this amino acid was the predominant C terminus. Only 12% (360 cpm) of the radioactivity was detected in glycine. Tritium incorporation into non-C-terminal carboxymethylcysteine was suppressed as shown with synthetic CM[Gly[15]]SS. On the basis of incorporation experiments with synthetic CM-SS-28, it was estimated that 85–90% of the peptide in the 4K CM-SS fraction could be accounted for by CM-SS-28.

Sequence analysis of 4K SS did not indicate whether the cysteine residues 17 and 28 formed a disulfide bridge. However, since carboxymethylation of 4K SS to 4K CM-SS required precedent reduction as observed by reverse-phase high-pressure LC (monitored by RIA S-201), it was assumed that the two cysteine residues of 4K SS probably formed an intramolecular disulfide bridge.

4K CM-SS was eluted at the same retention time as CM-SS-28 containing methionine sulfoxide instead of methionine (Figure 1). This derivative of CM-SS-28 was detected as a side product of the carboxymethylation of SS-28 and identified by amino acid analysis after alkaline hydrolysis (4 M LiOH) preserving methionine sulfoxide. Since free methionine sulfoxide was converted to methionine in the presence of CM-SS-28 under the hydrolytic conditions applied to 4K CM-SS, it was concluded that the amino acid analyses of 4K CM-SS did not exclude the possibility that this SS-like polypeptide was obtained in the methionine sulfoxide form. The sequencing procedure used here did not discriminate between methionine and methionine sulfoxide either, as demonstrated by sequence analysis of synthetic gastric-releasing peptide containing methionine sulfoxide. By reverse-phase high-pressure LC, only PTH-methionine was detected (Märki et al., 1981).

It was reported (Patzelt et al., 1980) that rat pancreatic pro-SS may contain D-mannose and D-glucosamine. Since the presence of carbohydrate in 4K SS also could explain the more hydrophilic properties of 4K SS compared to SS-28, partially purified 4K SS (step 1, Table I) was subjected to affinity chromatography on concanavalin A–Sepharose columns. No binding of 4K SS could be observed, excluding the presence of D-mannopyranose, D-glucopyranose, or their derivatives in 4K SS.

Sequence analysis had revealed that 4K SS contained the SS structure linked to the residual polypeptide through a pair of basic amino acids, arginine (residue 13) and lysine (residue 14). The selectivity of trypsin cleavage at this site of 4K SS was investigated. Digestion of 4K SS by TPCK-treated trypsin was monitored by RIA S-201 and RIA S-39. When ovine hypothalamic 4K SS partially purified (step 1, Table I) was digested with low concentrations of TPCK-trypsin, immunoactivity in RIA S-39 appeared (Figure 2), indicating cleavage of the peptide bond between residues 14 (lysine) and 15 (alanine). The activity in RIA S-39 increased only slowly, did not reach the original activity in RIA S-201 within 20 h of incubation, and was accompanied by a decrease of immunoactivity in RIA S-201, probably caused by hydrolysis of peptide bonds within the SS structure. Thus far, we have been unable to establish digestive conditions (by varying the enzyme concentration and thereby the substrate/enzyme ratio) which generated immunoactivity in RIA S-39 without diminishing the immunoactivity in RIA S-201. We had earlier been able to establish such conditions for rat hypothalamic 12K SS (Spiess & Vale, 1980).

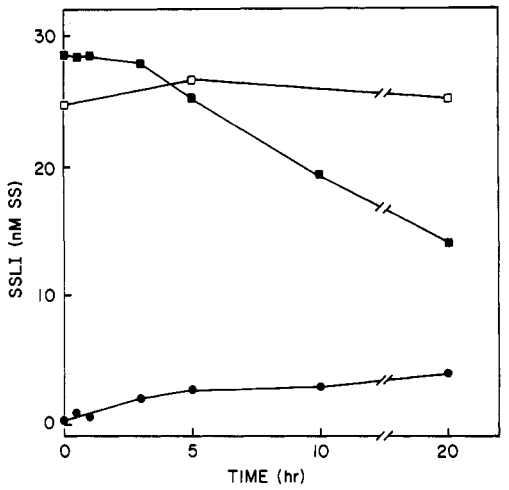

FIGURE 2: Time course of SSLI in RIA's S-201 (■) and S-39 (●) of ovine hypothalamic 4K SS (46 pmol of SS equivalents in RIA S-201, 24 μg of protein) after addition of TPCK-trypsin (typical experiment). The weight ratio of enzyme to protein substrate was 1:3500. Control incubations were performed in the absence of TPCK-trypsin and monitored by RIA S-201 (□).

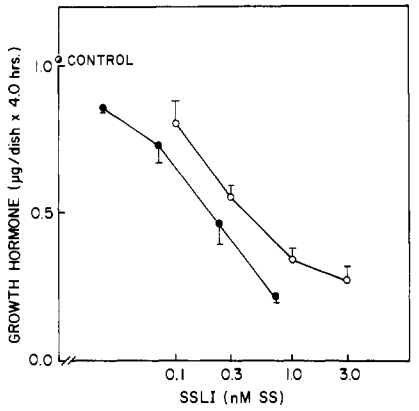

FIGURE 3: Inhibition of IBMX-stimulated growth hormone release from cultured rat anterior pituitary cells by synthetic SS and ovine hypothalamic 4K SS (27 pmol of SS equivalents in RIA S-201) partially purified by ion-exchange chromatography. SSLI of 4K SS (●) and synthetic SS (○) was determined by RIA S-201. The control value indicates the IMBX-stimulated growth hormone release in the absence of 4K SS or SS. Each point of the dose-response curve represents three dishes. Deviations are given as SEM.

The biological activity of 4K SS was determined by the inhibition of growth hormone release from cultured anterior pituitary cells of the rat (Figure 3). The biological potency (with 95% confidence limits) was 198% (124–325%) of the immunological potency (RIA S-201).

Discussion

Ovine hypothalamic 4K SS, an SS-like polypeptide with an apparent molecular weight in the range 3000–4500, was purified by ion-exchange chromatography and reverse-phase high-pressure LC to near homogeneity as demonstrated by reverse-phase high-pressure LC, amino acid analysis, and sequencing. The purification could only be successfully accomplished after chemical modification to the carboxymethylated product.

On the basis of the results of amino acid analysis, sequencing, and C-terminal end-group determination, it is suggested that ovine hypothalamic 4K SS is identical with SS-28, an SS-like polypeptide isolated from porcine upper small intestine and sequenced by Pradayrol et al. (1980).

The data of sequencing and amino acid analysis alone did not allow such a conclusion since one extra glycine in the composition of 4K CM-SS suggested the possibility that 4K SS could possess the structure of SS-28 C-terminally extended by glycine as discussed earlier (Spiess et al., 1980a,b). This possibility would have been compatible with the sequencing results since C-terminal amino acids are often lost by spinning cup sequencing. However, C-terminal analysis performed on a level of 200–700 pmol of peptide demonstrated that carboxymethylcysteine was the predominant C terminus. In view of these results, it appears probable that the extra glycine found by amino acid analysis belonged to a contaminating peptide (rich in glycine) which had escaped detection in the sequencer.

The observation that ovine CM-SS-28 was more hydrophlic than synthetic CM-SS-28 under the described conditions of reverse-phase high-pressure LC could be explained by reverse-phase high-pressure LC results, indicating that ovine CM-SS-28 was characterized in the methionine sulfoxide form. It has been described that methionine residues in peptides are easily oxidized in solution to sulfoxides by atmospheric oxygen (Savige & Fontana, 1977). It is therefore assumed that oxidation of the methionine residue of ovine SS-28 may have occurred during the isolation procedure.

Sequence analysis provided evidence that ovine SS-28 contained the SS structure linked through a pair of basic amino acids to the residual polypeptide. This structural element of multiple basic residues seems to be a characteristic cleavage site for the conversion of precursors into their hormones. Accordingly, ovine SS-28 was considered a possible SS precursor. The conversion usually requires trypsin-like activities which can be mimicked by trypsin in very low concentrations, as shown, for example, for the conversion of proinsulin and proparathyroid hormone into insulin and parathyroid hormone, respectively (Steiner et al., 1971; Goltzman et al., 1976). We had earlier reported that a small SS-like structure was selectively cleaved by TPCK-trypsin from rat hypothalamic 12K SS (Spiess & Vale, 1980). Immunological evidence suggests that we were unable to establish such selective digestive conditions for ovine SS-28. These results, therefore, do not support the candidacy of ovine hypothalamic SS-28 as an SS precursor.

Our finding that ovine hypothalamic SS-28 was biologically more potent than SS in inhibiting growth hormone release from cultured anterior pituitary cells is in agreement with reports from other laboratories. Vaysse et al. (1980) presented data of experiments with dogs, demonstrating that SS-28 was more potent than SS in inhibiting bombesin-stimulated glucagon release, whereas it was found equipotent with SS in inhibiting insulin, gastrin, and gastric acid release stimulated by bombesin. Subsequently, Brazeau et al. (1980) described that SS-28 was 3–14 times as potent as SS in the rat anterior pituitary cell culture assay. Thus far, it has not been established that the higher potency of SS-28 relative to SS is due to higher affinity for SS receptors or simply reflects greater stability of SS-28 under various bioassay conditions. Vaysse et al. (1980) have attributed the high potency of SS-28 to its prolonged action in vivo. In further in vitro experiments carried out with synthetic SS-28 under conditions where SS and SS-28 have equivalent stability, we have not found SS-28 to be more potent than SS (W. Vale, N. Ling, and J. Rivier, unpublished experiments). However, SS-28 is (like SS) a highly potent peptide, exhibiting biological effects at concentrations less than 10^{-10} M. In view of this high biological potency, it is possible that SS-28 itself possesses regulatory functions.

The biological importance of the partially sequenced polypeptide is unknown. Comparison with amino acid sequences of known polypeptides revealed only partial alignments, but

did not provide insight concerning the possible biological role of the discovered polypeptide. The existence of two pairs of basic amino acids suggests the possibility that this polypeptide represents a precursor which could be cleaved to smaller polypeptides.

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