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Primary Structure of the Ovine Hypothalamic Luteinizing Hormone-Releasing Factor (LRF)

(LH/hypothalamus/LRF/gas chromatography–mass spectrometry/decapeptide/Edman degradation)

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ABSTRACT The primary structure of ovine hypothalamic hypophysiotropic luteinizing hormone-releasing factor, LRF, has been established as pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 by hydrolysis of the peptide with chymotrypsin or pyrrolidone-carboxylylpeptidase and by analysis of the products by an Edman-dansylation sequencing technique, as well as by mass spectrometry of the derived phenylthiohydantoins. A decapeptide with the proposed primary structure, prepared by total synthesis, gave the same result on sequencing. The synthetic decapeptide possesses the same biological activities as the native ovine LRF. The amino-acid sequence of ovine LRF is identical to that already published for porcine LRF.

Various areas of the central nervous system participate in the fine regulation of the secretion of all adenohypophysial hormones. The ultimate integrator of information originating in the central nervous system is the hypothalamus. The final information from the hypothalamus to the adenohypophysis is not transmitted in the form of nerve impulses, but is carried in the form of specific hypothalamic hypophysiotropic substances, the hypothalamic releasing factors, that are carried through the hypothalamo–hypophysial portal system of capillaries from the median eminence region of the ventral hypothalamus to the cells of the adenohypophysis. There is good physiological evidence that such a hypothalamic control is involved in the secretion of the gonadotropin, luteinizing hormone. In the early 1960s, several investigators reported experimental results that were best explained by proposing the existence of substances that specifically stimulated the secretion of luteinizing hormone, and that were probably polypeptides, in crude aqueous extracts of hypothalamic tissues of various mammalian species (1–3). Preparations of LRF, active at 1 μg per dose in animal bioassays, were obtained by gel filtration and ion-exchange chromatography on carboxymethylcellulose (4), an observation that was confirmed by similar methods by several investigators (5, 6). In spite of the vagaries of the various bioassay methods available, several laboratories reported preparations of LRF of increased potency (5, 6). Several of these early publications led to contradictory statements regarding purification and separation of LH-releasing factor (LRF), from a follicle-stimulating hormone releasing factor (5, 7). Two laboratories independently reported the isolation of porcine LRF (8) and ovine LRF (9), both groups concluding that LRF from either species was a nonapeptide containing, on the basis of acid hydrolysis, 1 His, 1 Arg, 1 Ser, 1 Glu, 1 Pro, 2 Gly, 1 Leu, 1 Tyr. Earlier results with the pyrrolidone-carboxylylpeptidase prepared by Fellows and Mudge (10) had led us to conclude (11) that the N-terminal residue of LRF was Glu in its cyclized pyroglutamic (pGlu) form, as in the case of hypothalamic TRF, (pGlu-His-Pro-NH_2).

While our own studies on the amino-acid sequence of ovine LRF were in progress, Matsuo *et al.* (12) reported that porcine LRF contained one residue of tryptophan (Trp), in addition to the other amino acids earlier observed by acid hydrolysis. On the basis of a series of elegant experiments, including enzymatic hydrolysis with chymotrypsin and thermolysin and analysis of the partial sequences of their decapeptide by Edman degradation–dansylation and selective tritiation of C-termini, Matsuo *et al.* proposed the sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 for porcine LRF, a sequence that was confirmed by the same group using Edman degradation of a preparation of porcine LRF treated with a pyrrolidone-carboxylylpeptidase (13). Their studies were carried out with less than 200 nmol of peptide. They also stated that synthesis of that particular sequence gave a material with biological activity. We then reported synthesis by solid-phase methods of the decapeptide pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, which after isolation from the reaction mixture had the biological activity *in vivo* and *in vitro* of ovine LRF (14).

In this publication, we report the amino-acid sequence of ovine LRF obtained by analysis of hydrolysis products of LRF after digestion with chymotrypsin or pyrrolidone-carboxylylpeptidase, using Edman-degradation followed by determination of N- and C-termini by a quantitative dansylation technique. Confirmation of the positions of some of the amino-acid residues obtained by combined gas chromatographic–mass spectrometric analysis of phenylthiohydantoin (PTH) derivatives (15, 16) resulting from Edman degradations will also be described. We also report results obtained by degradation of synthetic LRF, since they confirm and clarify some peculiarities observed upon enzymatic cleavage of the native peptide.

A preliminary note describing these conclusions was published (17).

Abbreviations: LRF, luteinizing hormone releasing factor; Dns-, dimethylaminonaphthalene-5-sulfonyl-, (dansyl-); PTH, phenylthiohydantoin.

MATERIALS AND METHODS

The total amount of ovine LRF available for these studies was about 40 μg, or 30 nmol of peptide (measured by quantitative dansylation as described below); it was prepared as described by Amoss *et al.* (9) and represents about half of the total LRF yield from side fractions reserved from the early stages of the TRF isolation (18) from about 300,000 sheep hypothalamic fragments; the other half of the LRF fraction was used for the studies reported in ref 9.

Pure synthetic LRF decapeptide was prepared by solid-phase methods on a benzhydrylamine resin (14); the synthetic preparation had the biological activity *in vivo* and *in vitro* of ovine LRF.

Chymotrypsin A was 3× crystallized (Worthington, Lot CDI 8HD). Pyrrolidone-carboxylylpeptidase was obtained as described by Fellows and Mudge (10). Reagents used were reagent grade or analytical grade, some being further purified as described in the text.

Dansylation. Dansylation (19) of amino acids and peptides was conducted using [^{14}C]Dns-Cl (19–21). The sample, 50–100 pmol of hydrolyzate for direct amino-acid determination, 1–1.5 nmol for N-terminal analysis of peptides, was incubated at 37°C for 1 hr with 10 μl of 50 mM aqueous $NaHCO_3$ buffer (pH 9.3) and 10 μl of 7.7 nmol/μl of [^{14}C]Dns-Cl (45 Ci/mol Schwartz) in acetone.

Hydrolysis. Peptides were hydrolyzed in 6 × 50 mm Pyrex ignition tubes with 50 μl of triple-distilled 6 N HCl containing 0.5% thioglycolic acid (22). Tube contents were degassed by freezing and thawing at 50–100 μm (of Hg) pressure, and the sealed tubes were then incubated for 20–24 hr at 110°C. The same hydrolysis procedure was used for dansylated peptides, except that the hydrolysis time was reduced to 7.5 hr. When Dns-Pro was expected, hydrolysis was for 5 hr. Hydrolyzates were concentrated to dryness at 5–10 μm (of Hg) over NaOH.

Separation of Dns-Amino Acids. [^{14}C]Dns-amino acids were separated by two-dimensional thin-layer chromatography on 15 × 15 cm polyamide-coated sheets (Gallard Schlesinger) (23). The solvent for the first dimension was water–90% formic acid 200:3, and for the second dimension, benzene–glacial acetic acid 9:1. To further separate Dns-His, Dns-Arg, and Dns-Ser from each other and from other radioactive zones due to excess reagent, additional chromatography on the same sheet was conducted with chloroform–ethanol–glacial acetic acid 114:12:9 or *n*-heptane–*n*-butyl alcohol–glacial acetic acid 3:3:1.

Fluorescent zones were cut from the chromatogram and their radioactivity was determined in 10 ml of scintillation fluid (Beckman BBS3 scintillation-cocktail) with a Beckman LS 233 Liquid Scintillation Counter. In these experiments, only those zones corresponding to products of the amino acids present in significant quantities in hydrolyzates of LRF, i.e., Glu, His, Trp, Ser, Tyr (as *di*-Dns-Tyr), Gly Leu, Arg, Pro, and $GlyNH_2$, were counted. Dns-NH_2 and *O*-Dns-Tyr were not routinely counted. Determination of [^{14}C]Dns-Pro and [^{14}C]Dns-Leu obtained by dansylation of Leu and Pro in hydrolyzates or standard mixtures of free amino acids gave an average of 28 ± 5 cpm/pmol for five determinations each.

Hydrazinolysis. Determination of C-Termini. C-terminal determination of peptides was by hydrazinolysis without deamidation, by a modification of the method of Mesrob and Hollyeyšovský (24). Aliquots of 1–1.5 nmol of peptide were treated at 110°C for 6 hr with 50 μl of anhydrous hydrazine (Matheson, Coleman and Bell) in 10 × 75 mm heavy-walled Pyrex ignition tubes sealed at 50–100 μm (of Hg), while the tubes were immersed in liquid nitrogen. The hydrazine was removed under reduced pressure over sulfuric acid, and the residues were dissolved in 30 μl of deionized water and 30 μl of benzaldehyde [Matheson, Coleman and Bell, washed with aqueous NaOH, dried over $CaCl_2$, and redistilled at 35°C, 1 mm (of Hg)]. The tubes were covered with paraffin film and, after the mixtures were allowed to stand for 15 min with occasional agitation on a vortex mixer, the benzaldehyde phase was drawn off with a 100-μl microsyringe. The process was repeated three times. The benzaldehyde phase was discarded, and the remaining (C-terminal) amino acids in the aqueous phase were determined by dansylation. The benzaldehyde extraction step was omitted for the determination of Trp C-termini in separate aliquots of sample.

Edman Degradation. Sequential degradation of peptides was by the method of Edman (25), as modified by Gray (19), in which the peptide is converted to the *N*-phenylthiocarbamyl derivative by treatment with phenylisothiocyanate in aqueous pyridine, and the N-terminal amino acid is cleaved from the rest of the peptide as the 5-thiazolinone by treatment with anhydrous trifluoroacetic acid. The thiazolinone is extracted from the aqueous solution by butyl acetate. Reagents and procedures used in these experiments were exactly as described (19), except that 50 μl/liter of ethanedithiol was included in the butyl acetate to help preserve the Trp and Ser derivatives for subsequent mass spectrometric analysis of PTH derivatives. Test tubes with Teflon-lined screw caps were used to avoid interference by paraffin film with mass-spectrometric examinations. N-terminal determinations by dansylation were done on aliquots of the aqueous phase.

Mass Spectrometry. Combination gas chromatography–mass spectrometry was used to confirm the identity of some of the residues in the peptide sequences. The butyl acetate phase from each Edman cycle was concentrated to dryness under reduced pressure on a rotary evaporator, treated with 300 μl of 1 N HCl (1 μl/ml ethanedithiol) for 1 hr at 80°C to convert the thiazolinones to the PTH derivatives, concentrated to dryness, and treated for 1 min at 80°C with 50 μl of *N,O*-bis-trimethylsilylacetamide, (Pierce). The trimethylsilylated PTH derivatives were injected into the port of a gas chromatograph (Varian Aerograph model 1740-1) equipped with a 152 × 0.31 cm (diameter) stainless-steel column packed with 3% SE-30 on 100/120 mesh Varaport (Varian Aerograph). The oven temperature was initially set at 180°C. After injection, the oven temperature was programmed to increase at the rate of 2°C/min for the first 20 min, then at 10°C/min until it reached 275°C; the temperature was held at 275°C for the remainder of the run. The helium flow-rate was set at a back pressure of 1.2 atm at the start of the injection. A Biemann-Watson separator was used to remove the helium carrier gas in order to increase the sample concentration before it entered the ion source of the mass spectrometer (Varian Mat CH-5). As the sample was injected into the gas chromatograph, the mass spectrometer was set to sweep in a repetitive scan mode from mass 30 to mass 500 at a resolution of about 500, so that one spectrum was collected and stored

TABLE 1. *Analysis of trimethylsilylated PTH derivatives**

PTH derivatives	Retention time (min)	Major peaks in the mass spectrum†
Gly	11.3	264 (M^+; 55), 249 (69), 86 (100)
Pro‡	13.6	232 (M^+; 55), 203 (23), 135 (75), 119 (41), 69 (72)
Leu	16.6	320 (M^+; 52), 305 (66), 277 (47), 264 (65), 249 (45), 142 (100)
Ser	18.5	366 (M^+; 49), 351 (35), 336 (65), 321 (56), 276 (30), 261 (32), 188 (18), 103 (74)
Tyr	30.9	442 (M^+; 5), 427 (16), 336 (55), 321 (9), 264 (16), 179 (100)
Trp	38.0	465 (M^+; 2), 450 (4), 336 (49), 321 (6), 202 (94)

* 2-μl aliquots of standard solution containing 0.5 μg of each of the PTH derivatives (Mann) in 1 μl were injected for gas chromatography–mass spectrometry.

† Numbers in parentheses denote percent height of the peak relative to the base peak.

‡ PTH-Pro does not have any active proton for silylation.

automatically on the magnetic tape of the computer (Varian 620/i) every 11 sec. The PTH derivative emerged from the gas chromatograph at its specific retention time, which served as a qualitative identification of the amino acid; the identity of the derivative was then confirmed by its mass spectrum (Table 1). The method is not applicable to His or Arg, which do not extract as the thiazolinones into butyl acetate; also, the sensitivity of the method for Trp derivatives is low.

Digestion with Chymotrypsin. A 15 nmol (about 20 μg) aliquot of LRF (ovine or synthetic) was incubated 18 hr at 37°C in 50 μl of 0.1 M NH_4OAc buffer (pH 8.1) with 1 μg of chymotrypsin A. After incubation, aliquots of the digest were taken for C- and N-terminal analysis, the remainder was dried under reduced pressure and dissolved in 50% pyridine for sequencing by the Edman-dansylation procedure.

TABLE 2. *C-terminal determination by hydrazinolysis and dansylation of ovine LRF, with and without chymotrypsin treatment**

	Net cpm				
Dns-amino acid†	Synthetic LRF	Ovine LRF	Chymotrypsin-treated Ovine LRF	Control	*n*
Gly	23,939	12,164	11,434	3807 ± 1599	3
His	1,848	62	12,691	1809 ± 433	3
Leu	127	391	1,371	2004 ± 1828	3
Pro	2,850	0	1,929	773 ± 160	3
Ser	0	0	3,850	1150 ± 826	3
Tyr/2	1,089	582	7,592	1610 ± 790	3
Trp	2,993	1,528	—	2605 ± 503	3
Trp (no benzaldehyde)	—	—	4,599	2653	

* All values represent net cpm/nmol sample, corrected for mean control values. Controls are expressed as mean cpm ± standard error for *n* controls.

† Tyr is determined as *O,N-di*-Dns-Tyr; thus, the value observed is divided by two.

Digestion with Pyrrolidone-Carboxylylpeptidase. LRF (15 nmol, 20 μg) was incubated with 3 μg of pyrrolidone-carboxylylpeptidase for 22 hr at 37°C in 40 μl of buffer (0.05 M potassium phosphate (pH 6.5)–0.03 M 2-mercaptoethanol–1 mM EDTA). The digest was then dried and subjected to sequence analysis. Control samples of each enzyme were incubated and carried through the C- and N-terminal analyses and sequencing procedures.

RESULTS

Determination by [^{14}C]dansylation of amino acids in ovine LRF after hydrolysis with 6 N HCl in the presence of thio-

TABLE 3. *Edman degradation plus dansylation of ovine LRF, with and without chymotrypsin treatment**

	Net cpm							
		Edman cycle after chymotrypsin						
Dns-amino acid	Untreated	0	1	2	3	4	Control	*n*
His	2995	—	—	—	—	—	—	—
Trp	0	8,177	0	66	911	2,828	1830 ± 418	6
Ser	240	26,249	1,692	—	400	3,684	1193 ± 538	3
Tyr/2	0	416	40,394	645	266	1,083	2332 ± 434	5
Gly	93	26,725	636	813	1,058	35,112	2266 ± 364	6
Leu	0	357	31,746	0	901	2,449	1580 ± 306	6
Arg	—	5,373	3,812	41,384	931	3,090	2033 ± 599	6
Pro	0	0	5,730	0	36,470	787	1540 ± 128	6
Gly-NH_2	—	—	—	—	—	34,339	3055	1
Total cpm	3328	67,297	84,010	42,908	40,937	49,033		
Total nmol amino acid†	0.1	2.4	3.0	1.5	1.5	1.8		

* Values of net cpm and controls were calculated as in Table 2.

† Calculated on the basis of 28 ± 5 cpm/pmol (see text).

TABLE 4. *Edman degradation plus dansylation of ovine LRF after treatment with PCA-peptidase**

Dns-amino acid	Net cpm Edman cycle									Control	*n*
	0	1	2	3	4	5	6	7	8†		
His	12,061	0	—	—	—	—	0	0	—	10,523 ± 6,394	6
Trp	0	4579	0	619	688	0	1141	2439	250	5,593 ± 1,262	8
Ser	0	713	10,483	0	0	191	0	0	—	2,954 ± 415	12
Tyr/2	619	233	2,682	3762	318	—	0	0	—	1,606 ± 213	12
Gly	4,604	0	0	209	8,430	0	0	2112	0	4,688 ± 699	12
Leu	0	3191	0	56	3,289	3710	454	0	0	713 ± 128	12
Arg	1,858	—	3,011	896	1,814	4591	4078	0	—	1,904 ± 615	7
Pro	0	0	0	2144	56	0	1517	2917	0	1,045 ± 264	12
Gly-NH_2	—	—	—	—	—	—	—	—	1625	1,191 ± 686	2
Total	19,142	8716	16,176	7686	14,595	8492	7190	7468	1922		
Total nmol amino acid	0.7	0.3	0.6	0.3	0.5	0.3	0.3	0.3	0.1		

* Legend as in Table 3, except that values for His and Arg were calculated by use of the appropriate control from each corresponding Edman cycle, because the cpm for these amino acids increased significantly with each cycle.

† Unhydrolyzed.

glycolic acid confirmed the presence of His, Arg, Ser, Glu, Pro, Gly, Leu, and Tyr, as reported earlier by Amoss *et al.* (9) and Schally *et al.* (8) for ovine and porcine LRF, respectively, plus the presence of Trp, as originally reported by Matsuo *et al.* (12) for porcine LRF.

Chymotryptic digestion of ovine LRF gave four major peptide fragments having as C-termini (Table 2): Gly, His, Trp, Tyr, and possibly a small amount of a peptide with C-terminal Ser. Hydrazinolysis–dansylation showed C-terminal Gly in untreated ovine and synthetic LRF. Since this Gly residue is amidated in the synthetic preparation, and was shown by sequencing to be amidated in ovine LRF (see below), there was probably some deamidation of the Gly-NH_2 residue during the hydrazinolysis procedure. Dansylation of the chymotryptic digest of ovine LRF revealed primarily N-terminal Ser and Gly, plus some Trp and Arg (Table 3). The major N-termini observed after the first Edman cycle were: Tyr and Leu, plus some Arg and Pro; after the second cycle: Arg; the third: Pro; and the fourth: Gly-NH_2; Gly-NH_2 was determined without hydrolysis by 6 N HCl as Dns-Gly-NH_2 (first dimension of polyamide thin-layer chromatography, R_f = 0.72; second dimension, R_f = 0.42), and, after hydrolysis, as Dns-Gly. Total recoveries of Dns-amino acids after each cycle were in good agreement with theoretical values. Mass spectral analysis of PTH derivatives showed Gly and Ser (trace of Leu) from the first Edman cycle and Pro from the fourth Edman cycle; the extract from the second Edman cycle was accidently destroyed; to be consistent with the dansylation results the third cycle should have given Arg, which is not detectable by this method. These results are consistent with the primary structure for LRF shown in Fig. 1, the major fragments from chymotryptic digestion being Ch I, Ch II, Ch III, and Ch IV, with smaller amounts of Ch V and Ch VI. Hydrolysis of peptides by chymotrypsin at His-, Ser-, and Leu-residues is not uncommon (26). The same principal cleavages occur upon digestion of synthetic LRF with chymotrypsin. The results of analysis of the chymotryptic digest do not rule out an alternative structure for ovine LRF, in which the Ser-4 and Gly-6 residues are interchanged; that is, pGlu-His-Trp-Gly-Tyr-Ser-Leu-Arg-Pro-Gly-NH_2. Moreover, the position of the His residue can only be obtained by inference, since it is not directly accounted for.

Digestion of ovine LRF with pyrrolidone-carboxylyl-peptidase, followed by the Edman–dansylation analysis (Table 4), however, confirms the sequence PCA I shown in Fig. 1. Again, synthetic LRF gave similar results. Consistent with the sequence shown in Fig. 1, mass-spectral analysis revealed trimethylsilylated PTH derivatives of Trp, Ser, Tyr, Gly, and Leu in the second to sixth Edman cycles, respectively, and Pro in the eighth cycle from pyrrolidone-carboxylylpeptidase-digested ovine LRF. Blanks occur in the first and seventh cycle, as would be expected, since the determination of His or Arg is not possible by the technique described above. All of the remaining degraded peptide after the eighth Edman cycle was dansylated because of the limited amount of material, so that the identity of the tenth residue could not be corroborated by our gas-chromatographic method. In contrast to the results with chymotrypsin, the total recoveries of Dns-amino acids were lower than expected after pyrrolidone-carboxylylpeptidase digestion of either ovine or synthetic LRF. Furthermore, there was some evidence for endopeptidase activity in the pyrrolidone-carboxylylpeptidase preparation used that gave

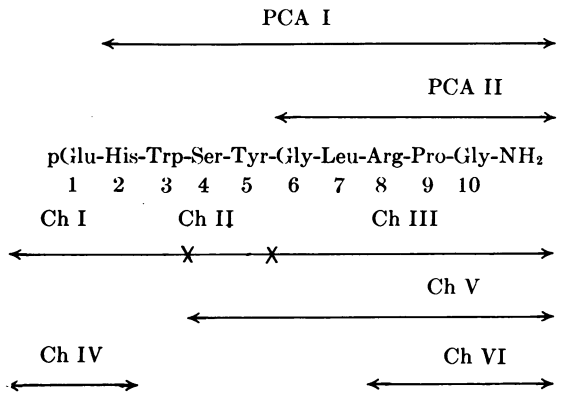

FIG. 1. Peptide fragments observed after treatment of ovine or synthetic LRF with chymotrypsin (Ch I–VI) or pyrrolidone-carboxylylpeptidase (PCA I–II).

rise to the fragment PCA-II, similar to Ch III (Fig. 1), since there is no evidence for this fragment in the undigested LRF preparations (Table 3). The batch of pyrrolidone-carboxylyl-peptidase used in these experiments does show more rapid loss of enzymatic activity upon storage than do batches of the enzyme used in our previous experiments (11) and, therefore, may contain some endopeptidase activity (R.F., unpublished data).

DISCUSSION

The amino-acid sequence of the hypothalamic releasing factor for luteinizing hormone is apparently the same in two different species (pig and sheep). A similar relationship has already been shown to exist in the case of the hypothalamic thyrotropin releasing factors of ovine and porcine origin, both of which have the amino-acid sequence pGlu-His-Pro-NH_2 (27–29). Of considerable physiological interest is the observation (12, 14, 30) that synthetic LRF stimulates the secretion of both luteinizing and follicle-stimulating hormones, thus reducing the probability that the follicle-stimulating hormone released by native LRF might have been due to a contaminant. These results do not settle the question of the possible existence of a follicle-stimulating hormone-releasing factor different from LRF (for a discussion of this point, see ref. 9).

LRF of ovine or porcine origin is biologically active in stimulating secretion of gonadotropins in several mammalian species (5, 6), including man (31). Knowledge of the structure of LRF from these species allows its synthesis in large quantities for possible use in testing pituitary capacity for gonadotropin secretion and as a means of inducing ovulation in farm animals, as well as in human beings. Synthetic analogues of LRF antagonistic to LRF would also be of interest as possible means of fertility control.

NOTE ADDED IN PROOF

After our initial communication describing chemical and biological properties of the purified LRF decapeptide synthesized on a benzhydrylamine solid support (See ref. 14, above), notes from other laboratories have appeared describing other routes of synthesis of the decapeptide: Sievertsson, H., Chang, J. K., Bogentoff, C., Currie, B. L., Folkers, K. & Bowers, C. Y., *Biochem. Biophys. Res. Commun.*, **44,** 1566–1571 (1971); Geiger, R., König, W., Wissman, H., Geisen, K. & Enzmann, F., *Biochem. Biophys. Res. Commun.*, **45,** 767–773 (1971); Matsuo, H., Arimura, A., Nair, R. M. G. & Schally, A. V., *Biochem. Biophys. Res. Commun.*, **45,** 822–827 (1971).

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