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Primary structure of corticotropin-releasing factor from ovine hypothalamus

(micro-sequence analysis/reverse-phase high-pressure liquid chromatography/dansylation/thermolysin/carboxypeptidase Y)

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ABSTRACT **Sequence analysis was performed of an ovine hypothalamic 41-residue polypeptide that had been postulated to be a putative corticotropin-releasing factor (CRF) because of its high intrinsic corticotropin releasing activity. The NH_2-terminal 39 residues of CRF were determined by Edman degradation of 0.6–3.5 nmol of peptide in a Wittmann-Liebold modified Beckman 890C spinning cup sequencer with reverse-phase high-pressure liquid chromatography for the identification of amino acid phenylthiohydantoins (direct micro-sequence analysis). Evidence for residue 40 (isoleucine) was provided by direct micro-sequence analysis of 2.0 nmol of acetylated CRF selectively cleaved at its arginine residues by trypsin prior to analysis. The thermolytic COOH-terminal fragment isoleucyl-alanineamide was characterized as its dansyl derivative. Based on the analytical data, the following primary structure is proposed for ovine hypothalamic CRF: H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2. In agreement with this proposal, the synthetic replicate of CRF is highly potent in stimulating secretion of both corticotropin and β-endorphin-like immunoactivities.**

Direct evidence for a hypothalamic corticotropin (ACTH) releasing factor (CRF) that stimulates ACTH secretion from the pituitary was first reported by Guillemin and Rosenberg (1) and Saffran and Schally (2) in 1955. Since that time, several groups have attempted to establish the chemical nature of this factor (see refs. 3 and 4 for review).

The efforts of this laboratory have been focused on a CRF fraction that originated from side fractions produced during the purification of luteinizing hormone-releasing factor (luliberin) from 490,000 ovine hypothalami (5) carried out in the Laboratories for Neuroendocrinology of the Salk Institute. This CRF fraction was selected for further purification because it exhibited the highest intrinsic ACTH releasing activity of all hypothalamic extracts and partially purified fractions assayed in primary cultures of dissociated rat anterior pituitary cells (6). In view of the loss of this activity after digestion with trypsin, it was suggested that this CRF was a polypeptide.*

The purification of ovine hypothalamic CRF (7), monitored by the rat anterior pituitary cell culture assay (6), included extraction with organic solvent systems, ultrafiltration, gel filtration, ion exchange chromatography, and (8) reverse-phase high-pressure liquid chromatography (RP-HPLC). The gel filtration experiments did not provide conclusive information about the apparent molecular weight of CRF.

We present here details of the determination of the primary structure of ovine hypothalamic CRF which was performed with a total of approximately 90 μg of peptide.

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EXPERIMENTAL PROCEDURES

Materials. CRF and several CRF analogs were synthesized by using solid-phase methods. [$MetO^{21}$]CRF was generated from CRF by oxidation with H_2O_2. Details of the CRF synthesis will be described elsewhere. H-Ile-Ala-OH and alanineamide were gifts from Bachem Fine Chemicals (Torrance, CA). Bovine insulin and thermolysin were purchased from Calbiochem. Bovine TPCK-trypsin and carboxypeptidase Y were purchased from Worthington. All other polypeptides were synthesized in this laboratory. [^{3}H]H_2O (5 Ci/ml; 1 Ci = 3.7×10^{10} becquerels) was obtained from Amersham or New England Nuclear. 3-Sulfophenylisothiocyanate, purchased from Pierce, was twice crystallized from water. All organic solvents were redistilled.

Amino Acid Analysis. Peptides (0.2–0.5 nmol) were hydrolyzed with 4 M methanesulfonic acid as described (9, 10) or with 50 μl of 6 M HCl plus 3 μl of thioglycol per ml containing norleucine as internal standard (110°C for 24 hr or 140°C for 3 hr). The hydrolysate was dried and worked up as described (10).

Protein Determination. Protein was determined by amino acid analysis.

COOH-Terminal Tritiation. Peptides (0.1–0.3 nmol) were tritiated according to Matsuo and Narita (11). Norleucine was added as internal standard before the start of the tritiation. Tritiated peptides were hydrolyzed with 6 M HCl plus 3 μl of thioglycol per ml (110°C for 24 hr) and subjected to amino acid analysis and scintillation counting as suggested by Cappugi *et al.* (12).

Spinning Cup Sequence Analysis. Edman degradation of peptides was performed with a Wittmann-Liebold modified Beckman 890C spinning cup sequencer as described (10). A few changes were introduced. Solvent S3 used for the extraction of the 2-anilino-5-thiazolinone derivatives of amino acids consisted of 0.3 mM dithioerythritol in *n*-butylchloride containing 15% (vol/vol) acetonitrile. The cleavage time was extended from 300 to 400 sec. The 3-phenyl-2-thiohydantoin (>PhNCS) derivatives of the amino acids formed in the converting flask were collected in tubes containing >PhNCS norleucine as internal standard. The repetitive yield was usually >95%. Carry-over ranged from 6% to 10%.

Trypsin Digestion of Acetylated CRF and Subsequent Sequence Analysis. Natural CRF or synthetic CRF analogs (approximately 2.0 nmol) were acetylated by the COOH-terminal tritiation protocol. The acetylated peptide was digested (1.5 hr

Abbreviations: ACTH, corticotropin; CRF, corticotropin releasing factor; >PhNCS, phenylthiohydantoin; RP-HPLC, reverse-phase high-pressure liquid chromatography.

* In compliance with the suggestions of several international nomenclature committees to use trivial names for biologically active polypeptides, we propose to name the polypeptide described "amunin" (from the Greek word ἀμύνειν, to ward off) to indicate its potential stress-related role in the acute defense of homeostasis.

at 37°C) with TPCK-trypsin in 0.05 M Quadrol trifluoroacetate/5 mM $CaCl_2$, pH 8.0. The substrate-to-enzyme weight ratio was 11:1. The mixture was directly applied to the sequencer cup.

End-Group Determination with Carboxypeptidase Y. Natural and synthetic CRF (final concentration, 8–10 μM) were digested at 37°C with carboxypeptidase Y in 0.1 M imidazole/5 mM EDTA/5% (vol/vol) *n*-propanol containing norleucine as internal standard. The pH was adjusted with acetic acid to 6.0 or 7.1. After defined time intervals, aliquots were removed, lyophilized, and analyzed with the amino acid analyzer. The amino acid concentrations were corrected on the basis of control experiments performed in the absence of substrate or enzyme or both.

Thermolysin Digestion of CRF. Peptide (0.20–0.35 nmol) was incubated with thermolysin (30°C for 22 hr) in 30 μl of 0.2 M *N*-ethylmorpholine/2 mM $CaCl_2$/5% (vol/vol) *n*-propanol, pH 8.0. The substrate-to-enzyme weight ratio was approximately 3:1. The mixture was lyophilized and dansylated in a procedure similar to the method recommended by Gray (13) for the dansylation of proteins. Dansyl amino acids or dansyl peptides carrying blocked carboxylic groups were extracted with chloroform and prepared for RP-HPLC. Control experiments were carried out in the absence of thermolysin or substrate or both.

Bioassay. CRF activity was assayed by the ability to stimulate the secretion of ACTH and β-endorphin-like immunoactivity from cultured rat anterior pituitary cells (6, 7).

RESULTS

CRF fractions prepared by different RP-HPLC procedures (8) were characterized by amino acid analysis. When aliquots of one of the CRF fractions were hydrolyzed with methanesulfonic acid under conditions that preserve all frequently occurring amino acids, the molar amino acid ratios normalized to four alanine residues deviated from integer numbers by an average of 8% (Table 1, column A). No tryptophan or cystine was detected. The relative glycine content was lower in the hydrolyzate of this CRF fraction than in the hydrolyzate of any other CRF fraction. All other amino acid ratios were in agreement with the corresponding values obtained from other CRF fractions hydrolyzed with HCl (Table 1, column B) or methanesulfonic acid. Extension of the duration of the hydrolysis with 6 M HCl from 24 to 72 hr did not increase the release of isoleucine, leucine, or valine.

Table 1. Amino acid composition of ovine hypothalamic CRF

Amino acid	Acid hydrolysis*		Sequence analysis
	A†	B‡	
Asp	—	—	3
Asn	—	—	1
Asx	3.8 (4)	4.0 (4)	—
Thr	1.8 (2)	1.8 (2)	2
Ser	2.9 (3)	3.0 (3)	3
Glu	—	—	3
Gln	—	—	4
Glx	7.1 (7)	7.0 (7)	—
Pro	2.1 (2)	2.1 (2)	2
Gly	0.3 (0)	0.9 (1)	0
Ala	4.0 (4)	4.3 (4)	4
Val	0.9 (1)	1.4 (1)	1
Met	1.0 (1)§	0.9 (1)	1
Ile	1.6 (2)	1.6 (2)	2
Leu	7.5 (8)	8.0 (8)	8
Tyr	0.1 (0)	0.3 (0)	0
Phe	0.7 (1)	0.9 (1)	1
Lys	2.1 (2)	2.1 (2)	2
His	1.9 (2)	1.8 (2)	2
Arg	1.9 (2)	2.1 (2)	2

* Amino acid ratios are presented; the nearest integer is given in parentheses.

† Peptide (1.0 μg) was hydrolyzed with 4 M methanesulfonic acid/0.2% tryptamine (110°C for 24 hr). The values are means of two analyses of one RP-HPLC-purified product. The coefficient of variation varied from 1% to 10% (except tyrosine, 38%).

‡ Peptide (0.4–0.8 μg) was hydrolyzed with constant boiling HCl containing thioglycol and norleucine (110°C for 24 hr). The values are means of a total of four hydrolyses of three separate RP-HPLC-purified products. The coefficient of variation was 3% to 12% (except glycine, 24%).

§ Determined as methionine sulfoxide.

Methionine was usually identified as such. However, some hydrolyses with methanesulfonic acid (Table 1, column A) yielded methionine sulfoxide instead of methionine. In view of the observation that methionine sulfoxide is most often converted to methionine under the hydrolytic conditions used here, the finding of methionine sulfoxide suggested that the methionine residues were oxidized in the starting material.

Amino acid analysis revealed that the CRF fractions were not homogeneous. However, to avoid losses, further purification of these fractions was not attempted. Because one of the analyzed CRF fractions contained only minor amounts of glycine, it was suggested that glycine was probably not part of the CRF molecule.

Direct micro-sequence analysis of natural CRF with a modified spinning cup sequencer was accomplished with a total of 44 μg of peptide. In each experiment, 2.9–16.8 μg of peptide (corresponding to approximately 0.6–3.5 nmol of CRF) was used. When 2.9 μg of peptide was applied to the cup, the NH_2-terminal 27 residues were identified.

The crucial experiment of sequence determination was performed with 16.8 μg of peptide modified with 3-sulfophenylisothiocyanate (14, 15) in the first sequencer cycle to improve the binding to the positively charged Polybrene film. Residues 1–39 of the main component, subsequently confirmed as CRF(1–39), were determined in this experiment (Fig. 1). The

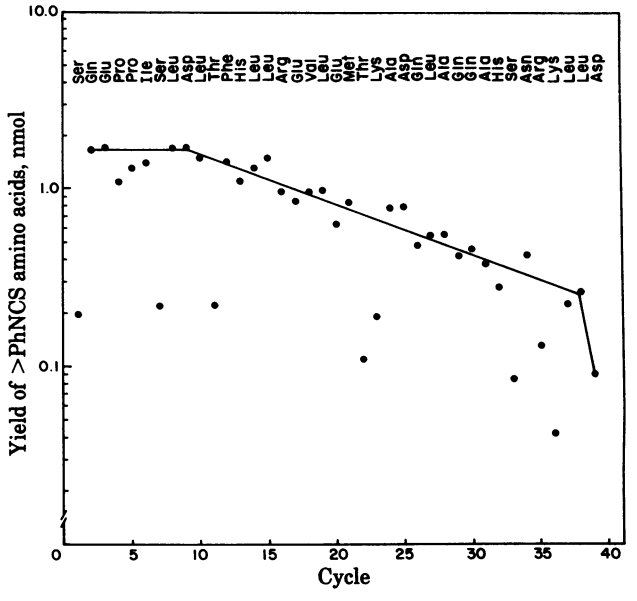

FIG. 1. Sequence analysis of ovine hypothalamic CRF (16.8 μg; 3.5 nmol). CRF was applied to the cup containing 6 mg of Polybrene and coupled to 1.2 μmol of 3-sulfophenylisothiocyanate in Quadrol. A second coupling was performed with phenylisothiocyanate. Cleavage in cycles 4 and 5 was extended (500 sec) and repeated once.

NH_2-terminal serine residue and the lysine residues were only partially modified with 3-sulfophenylisothiocyanate, as indicated by the yields of the >PhNCS derivatives of ε-*N*-phenylthiocarbamyllysine and -serine (Fig. 1). The relative >PhNCS amino acid yields of consecutive sequencer cycles were in agreement with the corresponding values determined with synthetic CRF and other peptides. No additional COOH-terminal amino acid residues could be detected. Extrapolation of the >PhNCS amino acid yields (from cycle 7 to cycle 38) to cycle 0 (Fig. 1) suggested that at least 3.0 nmol of natural CRF had been applied to the cup. This amount, corresponding to 12.7 μg of peptide [on the basis of the molecular weight of CRF(1–39)], represented approximately 76% of the total amount of protein applied to the cup. Therefore, CRF clearly was the main component of the CRF fraction used. Minor contaminants containing leucine, tyrosine, valine, and glycine were observed in all sequence experiments. On the basis of >PhNCS amino acid yields, they were estimated to represent 5–10% of the main component. Synthetic CRF(1–39) exhibited weak corticotropin releasing activity *in vitro* in contrast to CRF(1–27) which was not active.

In all sequence experiments, methionine-21 was identified as >PhNCS methionine and not as >PhNCS methionine sulfoxide as would have been expected on the basis of amino acid analysis. However, it has been demonstrated (16) that methionine sulfoxide is converted to methionine under the conditions of sequence analysis used here.

The residues identified by spinning cup sequence analysis accounted for 39 of the 41 amino acids found by amino acid analysis of CRF (under the assumption that CRF contained 7 Glx) (Table 1). The remaining two amino acids were alanine and isoleucine.

More insight into the COOH-terminal part of CRF was obtained by sequence analysis of 9.1 μg of peptide that was acetylated, cleaved by trypsin, and applied to the cup without purification. Only two fragments were degraded simultaneously—CRF(17–35) and CRF(36–41) (Fig. 2)—indicating a blocked NH_2-terminal fragment and selective tryptic cleavage at the COOH-terminal side of arginine-16 and -35. The only modified amino acids observed by sequence analysis were the lysine residues which were monitored as >PhNCS derivatives of ε-*N*-acetyllysine. The two CRF fragments behaved differently in the cup. The COOH-terminal fragment was lost significantly faster than CRF(17–35). The identification of both peptides thereby was facilitated because two distinct sets of >PhNCS amino acids were generated. The earlier sequence data were confirmed. In cycle 5 (equivalent to residues 21 and 40), 716 pmol of >PhNCS methionine, 80 pmol of >PhNCS isoleucine, and 48 pmol of >PhNCS valine were found. >PhNCS methionine was probably cleaved from CRF(17–35), whereas >PhNCS isoleucine or >PhNCS valine could be derived from the COOH-terminal fragment. Additional residues of the COOH-terminal fragment could not be detected although the conditions of sequence analysis were not impaired as demonstrated by the continuing degradation of CRF(17–35). On the basis of the amino acid composition of CRF and the sequence data, it was hypothesized that the COOH-terminal sequence of CRF was -Asp-Ile-Ala-NH_2 or -Asp-Ile-Ala-OH.

The consistency of the sequence data with this hypothesis was investigated by sequence analysis of synthetic CRF(33–41) and its COOH-terminal desamidated analog after acetylation and trypsinization. It was indicated by >PhNCS amino acid yields (Fig. 2) that the tryptic fragment of synthetic CRF(33–41) was degraded in a similar manner as the tryptic COOH-terminal fragment of natural CRF. The COOH-terminal alanineamide could not be detected. However, all residues of the COOH-terminal fragment of synthetic CRF(33–41) with a free terminal carboxylic group were identified by sequence analysis.

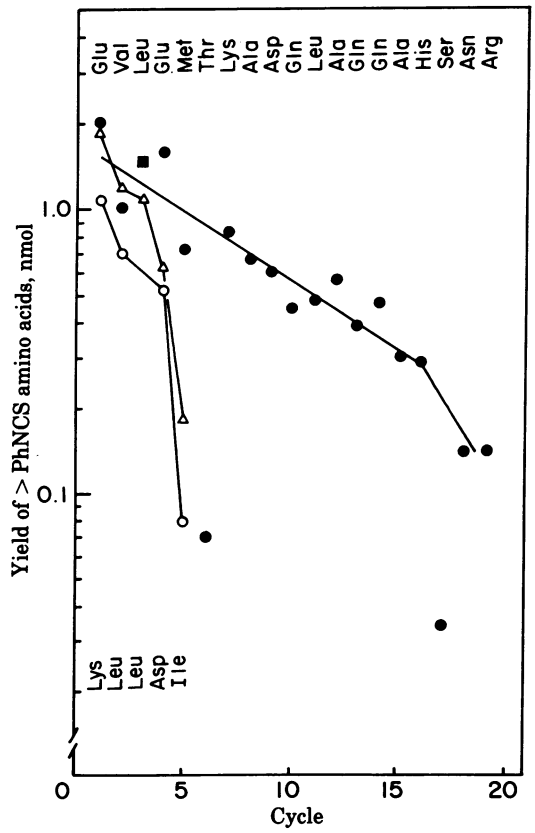

FIG. 2. Sequence analysis of the tryptic fragments of acetylated ovine hypothalamic CRF and synthetic CRF(33–41). Natural CRF (9.1 μg; 2.0 nmol) or synthetic CRF(33–41) (2.0 nmol) was acetylated, cleaved by trypsin, and applied to the cup containing 4 mg of Polybrene. Yields of the following fragments are presented: natural CRF(17–35) (●), natural CRF(36–41) (○), synthetic CRF(36–41) (△). >PhNCS leucine of cycle 3 (■) is derived from natural CRF(17–35) and natural CRF(36–41). Lysine residues were determined as >PhNCS-ε-*N*-acetyllysine. The structures of the CRF fragments are shown above and below the data points.

The COOH-terminal sequence of CRF was further investigated by digestion with carboxypeptidase Y which is known to cleave COOH-terminal amino acids with free or amidated carboxylic groups (17). The digested CRF fractions tested did not contain free amino acids with the exception of glycine which amounted to approximately 3% of the CRF content on a molar basis (assuming 7 Glx per CRF molecule). In view of the location of aspartic acid-39 in the COOH-terminal structure of CRF and the pH-dependent substrate specificity of the enzyme (17, 18), it was attempted to confine the digestion of CRF at pH 7.1 (upper pH limit of activity) to the amino acids on the COOH-terminal side of aspartic acid-39. After extensive digestion (2 hr, 37°C, enzyme/substrate ratio, 1:1.6), 0.85 nmol of alanine and 0.50 nmol of isoleucine were released per nmol of CRF (under the assumption of 7 Glx per CRF molecule). Only small amounts of leucine and aspartic acid were detected. A similar time course of amino acid release was found for synthetic CRF. These results suggested that the COOH-terminal sequence was -Ile-Ala-OH or -Ile-Ala-NH_2.

Under no conditions of carboxypeptidase Y digestion (at pH 6.0 or 7.1) was valine released. It therefore seemed improbable that residue 40 of CRF could be valine, although this would have been compatible with the sequence analysis data.

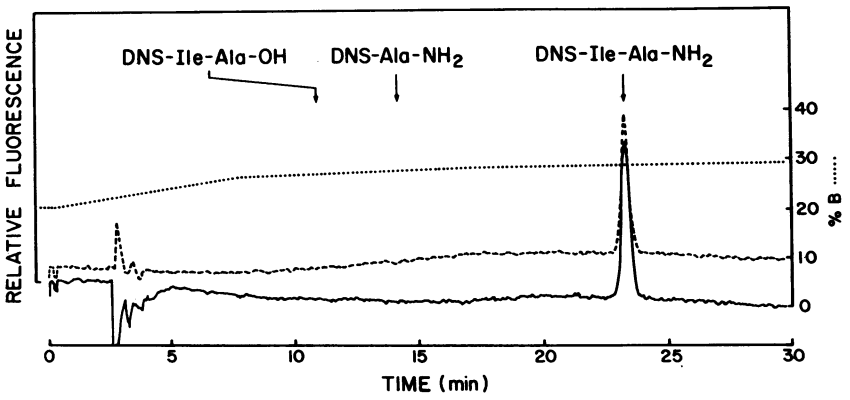

FIG. 3. RP-HPLC of synthetic dansyl isoleucylalanineamide (DNS-Ile-Ala-NH_2) and a dansyl derivative obtained from thermolytic digestion of natural CRF. A DuPont Zorbax ODS column (0.46 × 25.0 cm) was eluted at 50°C with a mixture of 0.08 M phosphoric acid, adjusted to pH 6.5 with triethylamine (A) and 35% (vol/vol) acetonitrile in *n*-propanol (B). The gradient of increasing % B is indicated. Excitation, 250 nm; emission, 500 nm. A Hewlett–Packard 1084B chromatograph equipped with a Schoeffel FS 970 spectrofluoro monitor was used. Separate runs with 1.9 pmol of synthetic DNS-Ile-Ala-NH_2 (----) and dansyl derivative of the natural product corresponding to 2.1 pmol of DNS-Ile-Ala-NH_2 (——) were carried out.

Based on the results of amino acid analysis, spinning cup sequence analysis, and carboxypeptidase Y digestion, the purity of CRF was estimated to be in the range 85–90%.

Whether natural CRF was COOH-terminally blocked was investigated by COOH-terminal tritiation. Only free terminal carboxylic groups are radioactively labeled by this procedure (11). No ^3H incorporation into alanine of natural or synthetic CRF was detected, whereas the specific radioactivities of the COOH-terminal alanine residues of synthetic CRF (with free terminal carboxylic group) and bovine insulin were 7060 and 11,140 cpm/nmol of alanine, respectively. On the basis of these results, it was excluded that alanine with free carboxylic group could be the COOH terminus of CRF. The tritiation results (together with the carboxypeptidase Y experiments) indicated that the COOH-terminus of natural CRF was alanineamide.

Direct evidence for the COOH-terminal amide of CRF was provided by characterization of thermolytic peptide fragments as dansyl derivatives by RP-HPLC with Zorbax CN and ODS columns (Du Pont). One of the dansylated CRF fragments was eluted from both columns with different solvent systems at the same retention time as synthetic dansyl isoleucylalanineamide (Fig. 3). Both products mixed prior to RP-HPLC were coeluted under high-resolving conditions of RP-HPLC (unpublished data) which separated all frequently occurring dansyl amino acids. Acidic hydrolysis of the purified dansylated fragment (6 M HCl for 4 hr at 110°C) generated dansyl isoleucine as the only dansyl amino acid as monitored by RP-HPLC. On the basis of these results, this fragment was identified as isoleucylalanineamide. No evidence for the presence of alanineamide in the thermolytic digest could be provided. The relative yield of dansyl isoleucylalanineamide prepared from natural or synthetic CRF was in the range 20–30%. Equivalent amounts of isoleucylalanineamide were cleaved from natural and synthetic CRF by thermolysin. It was concluded that this peptide represented the COOH-terminal sequence of CRF.

On the basis of the results of spinning cup sequence analysis, COOH-terminal analysis, and amino acid analysis, the following primary structure is proposed for ovine hypothalamic CRF: H-Ser-Gln-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met(O)-Thr-Lys-Ala-Asp-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2.

In aggreement with this proposal, natural CRF and its synthetic replicate [MetO^{21}]CRF did not differ significantly in RP-HPLC (7) and in their biologic potencies *in vitro*. Half-maximal responses of the secretion of ACTH and β-endorphin-like immunoactivity *in vitro* were estimated to be approximately 0.2 to 0.8 nM. Details of the biological and chromatographic characterization will be described elsewhere.

DISCUSSION

It was essential for the successful elucidation of the primary structure of CRF that the Beckman 890C spinning cup sequencer be modified according to Wittmann-Liebold (19, 20) to allow for direct micro-sequence analysis. This application of the modification was first described by Hunkapiller and Hood (21). It was found to be especially advantageous that, after modification, contamination with by-products of the Edman degradation was reduced and that sequence determination of more than 25–30 residues was not impaired because of increasing background caused by carry-over.

From amino acid analysis and RP-HPLC data, it was concluded that ovine hypothalamic CRF was obtained in the methionine sulfoxide form. In view of the observation that methionine residues of peptides can easily be oxidized in solution to the corresponding sulfoxides by atmospheric oxygen (22), it was probable that such oxidation had occurred during the purification of CRF. Accordingly, it is suggested that the primary structure of ovine hypothalamic CRF contains methionine rather than methionine sulfoxide. Synthetic CRF in the methionine form was found to be *in vitro* and *in vivo* more potent than [MetO^{21}]CRF (7).

The potency of synthetic CRF was determined to be of the same order of magnitude as the potency described for other hypophysiotropic peptides such as thyrotropin-releasing factor, luteinizing hormone-releasing factor, and somatostatin (6, 23).

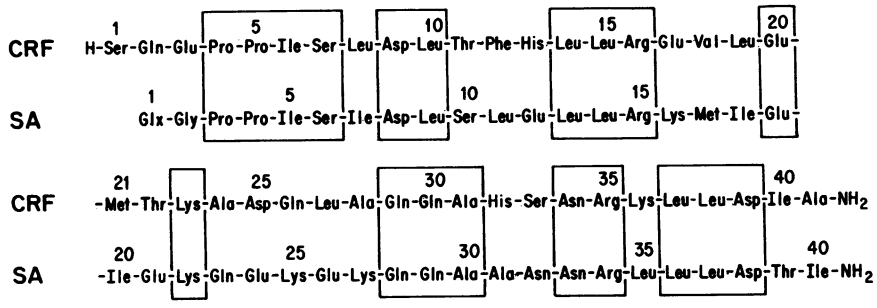

FIG. 4. Comparison of ovine hypothalamic CRF and sauvagine (SA). Alignments are marked by rectangles. Glx denotes pyrrolidonecarboxylic acid.

Ovine hypothalamic CRF has significant homologies with angiotensinogen (24) and sauvagine, a 40-residue polypeptide (from the skin of the frog *Phyllomedusa sauvagei*) characterized by Montecucchi *et al.* (25). The sequence of CRF from residues 12 to 15, -Phe-His-Leu-Leu-, is homologous with the sequence of residues 8 to 11 of angiotensinogen. In angiotensinogen, this group of amino acids represents the cleavage site for renin and converting enzyme (see ref. 26 for review) and may be a cleavage site in CRF as well. CRF and sauvagine align with 19 residues matching (Fig. 4). Sauvagine has been reported (27) to stimulate the release of ACTH and β-endorphin. The relative potencies of ovine hypothalamic CRF and sauvagine have not yet been determined.

At this time, the biologic meaning of the close structural relationship between the mammalian polypeptide CRF and the nonmammalian polypeptide sauvagine is unclear.

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1. Guillemin, R. & Rosenberg, B. (1955) *Endocrinology* **57**, 599–607.
2. Saffran, M. & Schally, A. V. (1955) *Can. J. Biochem. Physiol.* **33**, 408–415.
3. Saffran, M. (1977) in *Hypothalamic Peptide Hormones and Pituitary Regulation*, ed. Porter, J. C. (Plenum, New York), pp. 225–235.
4. Vale, W., Rivier, J. & Rivier, C. (1980) in *The Role of Peptides in Neuronal Function*, eds. Barker, J. L. & Smith, T. G., Jr. (Dekker, New York), pp. 432–454.
5. Burgus, R., Amoss, M., Brazeau, P., Brown, M., Ling, N., Rivier, C., Rivier, J., Vale, W. & Villarreal, J. (1976) in *Hypothalamus and Endocrine Functions*, eds. Labrie, F., Meites, J. & Pelletier, G. (Plenum, New York), pp. 355–372.
6. Vale, W., Grant, G., Amoss, M., Blackwell, R. & Guillemin, R. (1972) *Endocrinology* **91**, 562–572.
7. Vale, W., Spiess, J., Rivier, C. & Rivier, J. (1981) *Science*, in press.
8. Rivier, J., Rivier, C., Branton, D., Millar, R., Spiess, J. & Vale, W. (1981) in *Peptides: Synthesis, Structure and Function*, eds. Rich, D. H. & Gross, E. (Pierce Chemical, Rockford, IL), in press.
9. Spiess, J., Rivier, J., Rodkey, J. A., Bennett, C. D. & Vale, W. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2974–2978.
10. Spiess, J., Villarreal, J. & Vale, W. (1981) *Biochemistry* **20**, 1982–1988.
11. Matsuo, H. & Narita, K. (1975) in *Protein Sequence Determination*, ed. Needleman, S. B. (Springer, New York), pp. 104–113.
12. Cappugi, G., Nassi, P., Treves, C. & Ramponi, G. (1971) *Experientia* **27**, 237–239.
13. Gray, W. R. (1972) *Methods Enzymol.* **15**, 121–138.
14. Dwulet, F. E. & Gurd, R. N. (1976) *Anal. Biochem.* **76**, 530–538.
15. Braunitzer, G., Schrank, B. & Ruhfus, A. (1970) *Hoppe-Seyler's Z. Physiol. Chem.* **351**, 1589–1590.
16. Märki, W., Spiess, J., Tache, Y., Brown, M. & Rivier, J. E. (1981) *J. Am. Chem. Soc.* **103**, 3178–3185.
17. Hayashi, R. (1977) *Methods Enzymol.* **47**, 84–93.
18. Hayashi, R., Bai, Y. & Hata, T. (1975) *J. Biochem.* **77**, 69–79.
19. Wittmann-Liebold, B. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 1415–1431.
20. Wittmann-Liebold, B., Graffunder, H. & Kohls, H. (1976) *Anal. Biochem.* **75**, 621–633.
21. Hunkapiller, M. W. & Hood, L. E. (1978) *Biochemistry* **17**, 2124–2135.
22. Savige, W. E. & Fontana, A. (1977) *Methods Enzymol.* **47**, 453–459.
23. Vale, W., Brazeau, P., Rivier, C., Brown, M., Boss, B., Rivier, J., Burgus, R., Ling, N. & Guillemin, R. (1975) *Recent Prog. Horm. Res.* **31**, 365–397.
24. Skeggs, L. T., Jr., Kahn, J. R., Lentz, K. & Shumway, N. P. (1957) *J. Exp. Med.* **106**, 439–453.
25. Montecucchi, P. C., Henschen, A. & Erspamer, V. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 1178 (abstr.).
26. Marks, N. (1977) in *Peptides in Neurobiology*, ed. Gainer, H. (Plenum, New York), pp. 230–232.
27. Erspamer, V. & Melchiorri, P. (1980) *Trends Pharm. Sci.* **20**, 391–395.