Vol. 122, No. 3, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 16, 1984 Pages 899-905

ISOLATION AND CHARACTERIZATION OF THE BOVINE HYPOTHALAMIC CORTICOTROPIN-RELEASING FACTOR

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Received June 29, 1984

SUMMARY. A 41 amino acid peptide with high intrinsic corticotropin-releasing activity was isolated from 1000 bovine hypothalami by means of immunoaffinity chromatography, gel filtration, and two steps of reverse phase HPLC. The primary structure of the amino terminal 39 amino acids was characterized by gas phase sequence analysis. The sequence of the amidated carboxyl terminal dipeptide was established by digestion of the intact natural product with Staphylococcus aureus V8 protease, dansylation of the digest and comparative reverse phase liquid chromatography studies with the synthetic dansylated dipeptides Ile-Ala-NH_2, Ile-Ala-OH, Ala-Ile-NH_2 and Ala-Ile-OH. The complete structure of the bovine corticotropin-releasing factor was established as: 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-Asn-Asn-Arg-Lys-Leu-Leu-Asp-Ile-Ala-NH_2 using approximately 650 pmol of material.

The isolation, structural characterization and total synthesis of hypothalamic peptides which regulate the release of pituitary hormones have permitted the rapid accumulation of a wealth of knowledge in the regulation of the neuroendocrine system. The utilization of highly efficient polypeptide microisolation procedures (1) and ultrasensitive structural characterization capabilities, (2,3) have been of utmost importance in this regard. Recent improvements in purification protocols have included better extraction procedures (4) which yield a relatively protein-free peptide fraction, the use of affinity chromatography employing antibodies to the peptide or a related peptide of interest and high performance liquid chromatography (HPLC) on

ABBREVIATIONS:
HPLC = high performance liquid chromatography; RPLC = reverse phase liquid chromatography; >PhNCS = phenylthiohydantoin; bCRF, oCRF, rCRF and hCRF = bovine, ovine, rat and human corticotropin releasing factors, respectively; irCRF = immunoreactive corticotropin releasing factor; hGRF = human growth hormone releasing factor; TEAP = triethylammonium phosphate.

0006-291X/84 $1.50

preparative and analytical levels to achieve peptide homogeneity. Indeed, the availability of such efficient and resolutive methods allows the isolation of rare molecules using reasonably small amounts of starting material, i.e. a few grams of tissues rather than the thousands of grams necessary in the past. Recent advances in the microsequence analysis of polypeptides have included improvements in the reverse phase liquid chromatographic (RPLC) identification and quantitation of phenylthiohydantoin (>PhNCS) amino acids as well as the commercial availability of a powerful new instrument for automated Edman degradation, the gas phase microsequenator (3). We have recently begun to apply these powerful methodologies toward the isolation and structural analyses of hypothalamic corticotropin-releasing peptides from different species. This report describes the purification to homogeneity of the bovine hypothalamic corticotropin-releasing factor (bCRF) and its structural characterization by gas phase sequence analysis.

METHODS

Immunoaffinity chromatography. The non-retained fraction from approximately 1000 bovine hypothalamic fragments which failed to bind to an immunoaffinity column prepared by coupling purified IgG (5) raised against synthetic hGRF(1-40) (a fragment of the human growth hormone releasing factor) was used as the starting material for the purification of bCRF (6). This liquid was pumped through an immunoaffinity column (3.5 X 7.2 cm, V_{bed} = 70 ml) prepared by coupling purified IgG (5) raised against synthetic [Tyr^{21}]oCRF(21-41) to Affi-gel 10 (BioRad Laboratories) according to the manufacturer's recommendations. After washing off the unbound material with 20 mM sodium phosphate/145 mM sodium chloride/0.01% sodium azide, pH 7.4, the adsorbed immunoreactive CRF (irCRF) was eluted with 1 M acetic acid. The irCRF recovered from the affinity column was further purified by gel filtration and two steps of HPLC. Details of the purification procedure are given in the legend to Figure 1.

Radioimmunoassay, bioassay and structural characterization. During the peptide purification, column effluents were monitored for the presence of CRF by radioimmunoassay using an antiserum which was part of a pool of antisera used for immunoaffinity chromatography. Amino acid analyses (2) and gas phase sequence analyses were performed as previously described (3) except for the implementation of a new software program obviating the need for a vacuum system in the gas phase sequencer (designated 02NVAC and supplied by Applied Biosystems, Inc, Foster City, CA). Also, the resultant >PhNCS-amino acid residues were identified using an improved RPLC separation system suggested by W. Touchstone (Baylor University) and M. Hunkapiller (Applied Biosystems, Inc). Staphylococcus aureus V8 proteolytic digestion (7) of 100 pmols bCRF in 20 mM sodium phosphate, pH 7.8, was performed at 37°C in 15 hrs with a 1:35 mole enzyme:mole substrate ratio. The digest was reacted with dansyl chloride (8) and 10% of the reaction mixture (10 pmols) was subjected to reverse phase liquid chromatography as described in the legend to Figure 3 and detected with a Kratos/Schoeffel FS950 Fluoromat fluorescence detector.

RESULTS AND DISCUSSION

A four step purification procedure involving immunoaffinity chromatography, gel filtration and semi-preparative and analytical reverse phase HPLC was used to isolate bCRF. The high cross-reactivity between anti-oCRF(21-41) antibodies and bCRF made immunoaffinity chromatography a very efficient step for the purification of bCRF. Forty-five μg of immunoreactive CRF (irCRF) from 1000 bovine hypothalami were retained by the column and further purified as illustrated in Figure 1. Gel filtration (Figure 1A) yielded a single irCRF zone, which upon semi-preparative HPLC (Figure 1B), was separated into two irCRF fractions. Rechromatography of the major fraction on an analytical reverse phase HPLC system with different solute selectivity (Figure 1C) yielded two irCRF peaks, each corresponding to a distinct UV-light-absorbing peak of peptidic material. The major bCRF species was subjected to structural characterization and represented 7.6 nmol of peptide. The structure of the minor irCRF (Figure 1C) is unknown; it may represent a degradation product or it may be an oxidized form of bCRF.

The amino acid composition of the major irCRF (Table I) suggested a large degree of structural homology with ovine CRF (oCRF) (9,10). Direct Edman degradation of 500 pmol of intact bCRF by gas phase sequence analysis yielded the primary structure of the amino terminal 39 amino acids of the 41-residue peptide (Figure 2). Comparison with the amino acid composition of the intact bCRF showed that the remainder of the sequence could be narrowed down to one of four possible carboxyl terminal dipeptides: -Ile-Ala-NH_2, -Ile-Ala-OH, -Ala-Ile-NH_2 or -Ala-Ile-OH. These were synthesized by solid phase methodology (11), dansylated and used as standards for HPLC comparative studies with the dansylated Staphylococcus aureus V8 protease digestion products from 100 pmols of native bCRF. Figure 3 shows the elution positions of the dansylated synthetic dipeptides and the results of HPLC analysis of approximately 10 pmols of the dansylated bCRF digest: only synthetic dansyl-Ile-Ala-NH_2 elutes with a retention time identical to that of any of the dansylated digestion products. Hence, the complete structure of bCRF, including its amidated carboxyl terminus, was established with approximately 650 pmols of peptide. At this time the structures of peptides with high intrinsic

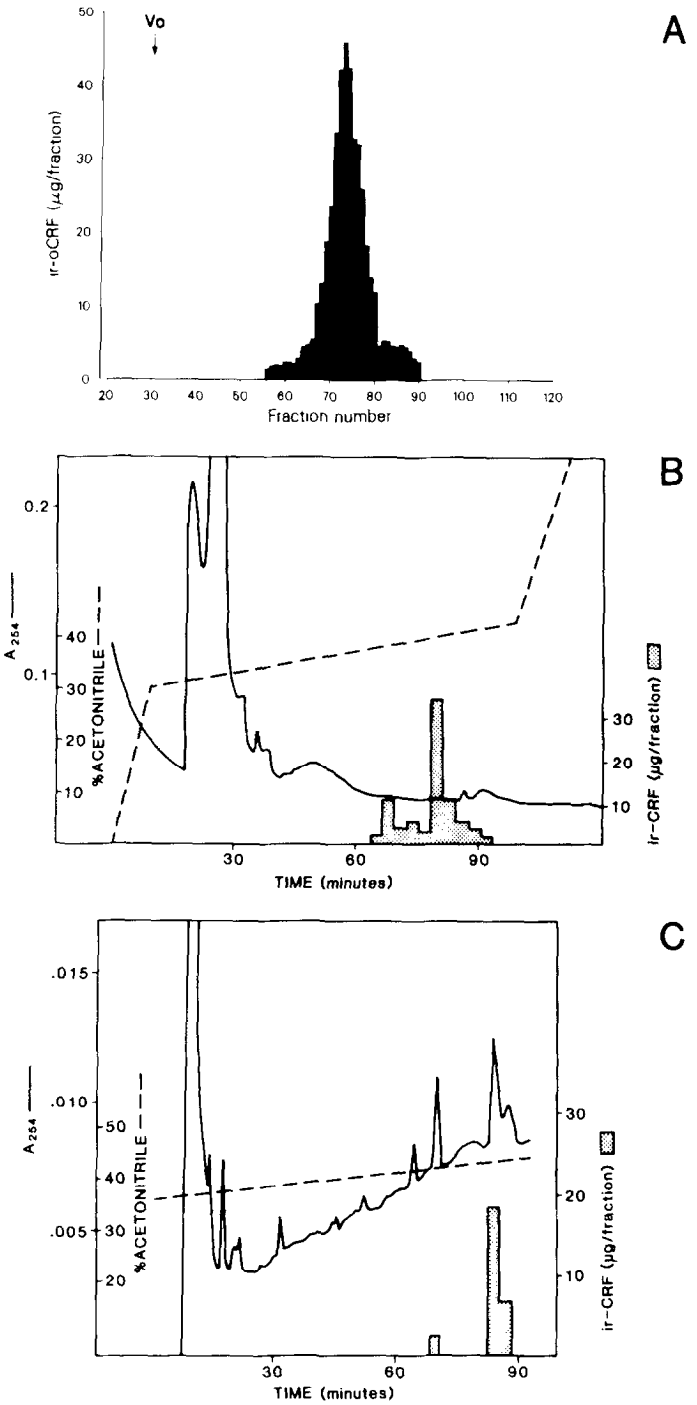

FIGURE 1: Isolation of bCRF.

A) The immunoaffinity-purified irCRF was chromatographed over a Sephadex G-75 column (4.5 x 117 cm) equilibrated and developed in 1 N acetic acid/0.2% 2-mercaptoethanol (v/v) at 1 ml/min.

B) The gel-filtered irCRF-containing fractions were pooled (fractions 67-80) and pumped onto a semi-preparative C18 column (Altex Ultrasphere, 5 µm particle size, 100 Å pore size, 1 X 25 cm)

TABLE I

AMINO ACID COMPOSITION OF bCRF[a]

Amino Acid	bCRF (n=2)	
Asx	4.98 ± 0.07	(5)[b]
Thr	1.91 ± 0.01	(2)
Ser	1.99 ± 0.12	(2)
Glx	6.92 ± 0.21	(7)
Gly	0.13 ± 0.03	
Ala	3.83 ± 0.01	(4)
Val	0.90 ± 0.01	(1)
Met	1.20 ± 0.01	(1)
Ile	1.93 ± 0.04	(2)
Leu	8.31 ± 0.15	(8)
Tyr	0	
Phe	0.94 ± 0.01	(1)
His	1.91 ± 0.10	(2)
Trp	0	
Lys	1.98 ± 0.06	(2)
Arg	2.07 ± 0.00	(2)
Cya[c]	0	
Pro	1.95 ± 0.25	(2)

a Values are means (± standard deviation) from n determinations and are not corrected for hydrolysis losses.

b Values in parentheses correspond to the integer values of the amino acids in bCRF.

c Cysteine was determined as cysteic acid.

corticotropin-releasing activity have been established from four mammalian species as shown in Figure 4. The ovine (9,10) and rat (12) CRFs were isolated and characterized from hypothalamic tissues while the human CRF structure was deduced from the cDNA encoding human CRF in a human genomic library (13).

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utilizing a 0.25 M triethylammonium phosphate (TEAP), pH 3.0/acetonitrile mobile phase. Fractions of 3 ml were collected at 1 ml/min.

C) The major irCRF species from step B was purified to homogeneity by chromatography on an analytical C8 column (Brownlee RP300, 7 μm particle size, 300 Å pore size, 0.46 x 25 cm) using a 0.2% (v/v) heptafluorobutyric acid (HFBA)/acetonitrile solvent system. Fractions of 3 ml were collected at 1 ml/min.

At all chromatography steps aliquots of column fractions were subjected to radioimmunoassay after drying in a vacuum centrifuge (Savant) in the presence of 100 μg serum albumin.

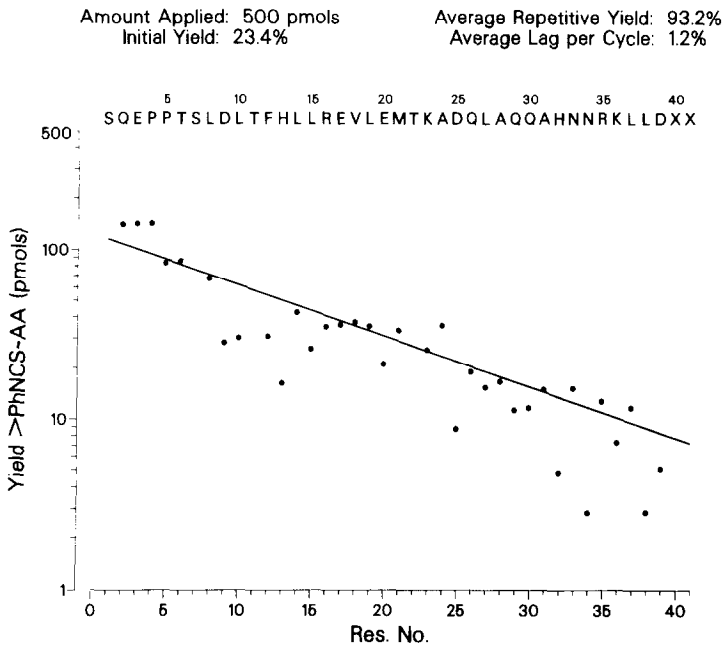

FIGURE 2: Gas phase sequence analysis of 500 pmol bCRF.

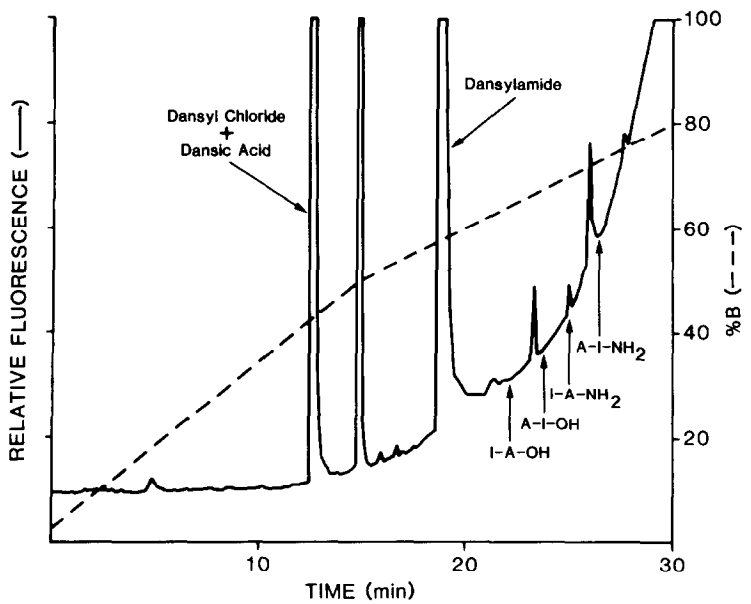

FIGURE 3: Reverse phase liquid chromatographic identification of the dansylated carboxyl terminal dipeptide of bCRF. Elution at 1 ml/min was accomplished with the indicated gradient at room temperature using a C18 column (Altex Ultrasphere, 5 μm particle size, 046 X 25 cm) and the following solvents: Solvent A, 0.25 M sodium acetate pH 5.9:MeOH:THF (80:19:1) and Solvent B, 20% solvent A and 80% methanol.

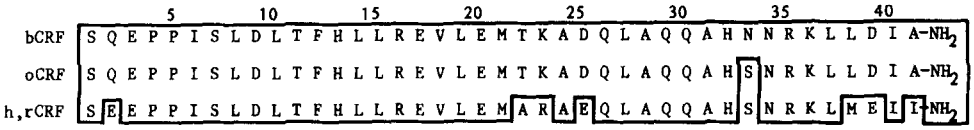

FIGURE 4: Primary structures of bCRF, oCRF, rCRF and hCRF. Sequence differences from the bovine structure are outlined.

ACKNOWLEDGEMENTS

We thank M. Mercado, R. Schroeder, F. Castillo, K. Von Dessonneck, R. Klepper M. Regno, D. Angeles and T.C. Chiang for their expert technical assistance. This research was supported by program grants from NIH (HD-09690 & AM-18811) and the Robert J. & Helen C. Kleberg Foundation.

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