Characterization of rat hypothalamic corticotropin-releasing factor
(peptide isolation/sequence microanalysis/peptide mapping/peptide synthesis)

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ABSTRACT A polypeptide was purified from rat hypothalamic extracts on the basis of its high intrinsic activity to release corticotropin (ACTH) from cultured rat anterior pituitary cells and its immunoreactivity in a radioimmunoassay directed against the NH2 terminus (residues 4-20) of ovine hypothalamic corticotropin-releasing factor (CRF). Based on Edman degradation, peptide mapping, and amino acid analysis, the primary structure of this rat CRF was established to be: H-Ser-Glu-Cys-Pro-Pro-Ile-Thr-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Ala-Arg-Ala-Glu-Leu-Ala-Glu-His-Ser-Glu-Leu-Asp-Leu-Thr-Ile-NH2. The hypothetical amino acid sequence of synthetic rat CRF did not deviate significantly from the structures of the isolated native peptide or of synthetic ovine CRF. The close structural relationship between rat and ovine hypothalamic CRF is indicated by an 83% sequence homology.

Harris proposed that the neuroregulation of adrenocorticotropic hormone (ACTH; corticotropin) may be mediated by a substance later termed corticotropin-releasing factor (CRF), which reaches the adenohypophysis by the hypothalamic-hypophyseal portal system (1). Early experimental observations by Guillemin and Rosenzweig (2), and Saffran and Schally (3), supported the presence of such factors in the hypothalamus that would increase the rate of ACTH secretion by the pituitary gland incubated in vitro or maintained in organ culture. More than 25 years later, ovine CRF (oCRF) was isolated (4, 5), characterized (5, 6), and synthesized (7). Subsequently the amino acid sequence of the protein precursor of oCRF was deduced from the cDNA (8). With the availability of synthetic oCRF and antibodies raised against it, various aspects of oCRF's distribution and of its hypo-physyal, vascular, and neural actions have been investigated (9-18), and immunoneutralization experiments in the rat with anti-oCRF antisera have demonstrated that this or related peptides are indeed involved in the physiologic regulation of ACTH secretion (19). As oCRF was purified from a side fraction (5) containing only a small percentage of the total ACTH-releasing activity in ovine hypothalamic extracts (20), it was necessary to establish the structure of the predominant CRF in fresh tissue extracts. Because of its importance as an experimental animal and because its CRF differed from oCRF with respect to immunological and chromatographic behavior, the rat was chosen as a source of CRF. We report here the isolation, characterization, and total synthesis of rat CRF (rCRF), a 41-residue peptide belonging to the family that includes oCRF, sauvagine (21), and urotensin I (22). In view of the fact that the latter three peptides have a spectrum of biological activities on the cardiovascular and central nervous systems of the mammal with differences in relative potencies (23-25), it was furthermore important to characterize CRF in the species in which its physiological functions have been most thoroughly investigated.

EXPERIMENTAL PROCEDURES

rCRF was purified from lyophilized rat hypothalamic fragments provided by A. Parlow under the aegis of National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. We used two separate biological tests to follow CRF activities throughout our purification schemes: (i) an in vitro method for assaying the ability of CRF to stimulate the secretion of ACTH and beta-endorphin by primary cultures of rat pituitary cells (5, 26) and (ii) a radioimmunoassay (RIA) with the C-24 antibody directed toward the NH2 terminus (residues 4-20) of oCRF and with HPLC-purified [125I]-labeled CRF, [Tyr22, Nle23]oCRF, or [Nle21, Tyr22]oCRF as the radioligand (22). C-24 CRF antibody was generated in a rabbit immunized with [Tyr22, Gly23]oCRF-(1-23) coupled to human a-globulin by bis-diazotized benzidine. Sensitivity of the assay is <1 fmol.

Peptides were made by the solid-phase approach. t-Butyloxycarbonyl N-tert protection (Boc) was used throughout. Side chains were protected as follows: benzyl esters (OBzI) for both aspartic and glutamic acids, benzyl ethers (OBzI) for both serine and threonine hydroxyl groups, xanththryl (Xan) for both asparagine and glutamine, tosyl (Tos) for both histidine and arginine, and chlorobenzyloxycarbonyl (2CIZ) for lysine. Couplings (1-2 hr) were meditated by dicyclohexylcarbodimide in CH3Cl2 or dimethylformamide as asparagine, glutamine, and arginine derivatives. A 3-fold excess of amino acid based on the original substitution in the residue was used. Double couplings were automatically performed at residues 14, 18, and 40. Syntheses were started with as many as 12 g of resin per run in a modified Beckman 990 B synthesizer. In that case, the peptide resin weighed 25 g and was cleaved in HF (150 ml) batchwise (8 g in the presence of anisole (20 ml) and methyl ethyl sulde (3 ml). Two trifluoroacetic acid (60% in CH3Cl2, 5% ethanediithiol) deblockings were performed at each step (10 and 15 min, respectively). An isopropyl alcohol (1% ethanediithiol) wash followed CF3COOH treatment prior to the two triethylamine (12% in CH3Cl2) neutralization steps (which included an intermediate MeOH wash). Each coupling step was followed by acetylation (10% CH3CO2O in CH3Cl2 for 15 min).

For purification of the synthetic peptides, cartridges fitting Waters Associates preparative liquid chromatograph 500 (LC-500) were packed with 15- to 20-µm C18, C4, or phenyl silicas (300 Å, Vydac). A gradient of CH3CN in TEAP buffer (27) was generated by a low-pressure Eldex gradient maker. Crude peptides (directly after HF cleavage and lyophilization) were loaded (3 g per run) through the pump; cuts were made, analyzed, and often rerun under different conditions until pure product was obtained. Desalting of the purified fractions, independently checked for purity, was achieved by using a gradient of CH3CN in 0.1% CF3COOH (28). The center cut was lyophilized to yield

Abbreviations: ACTH, adrenocorticotropic hormone or corticotropin; CRF, corticotropin-releasing factor; rCRF, rat CRF; oCRF, ovine CRF; RIA, radioimmunoassay; GH-RF, growth hormone (somatotropin)-releasing factor.
(10–15%) the desired product ([α]_D^22 = -93.7°, concentration = 1 g/100 ml in 1% AcOH after correction of peptide content by amino acid composition).

RESULTS AND DISCUSSION

Acetone defatting of the rat hypothalamic tissues and acid extraction followed by gel filtration on Sephadex G-50 has been described earlier in a scheme used for the initial purification of rat hypothalamic growth hormone (somatotropin)-releasing factor (GH-RF) (29). Several zones exhibiting CRF-like immunoreactivity were found. A high molecular weight zone appearing in the void volume of the gel permeation step (representing <10% CRF-like immunoreactivity) and a zone (representing ~90% CRF-like immunoreactivity) also containing GH-RF-like activity with a partition coefficient Kav of 0.20–0.31 were both biologically active as well as immunoreactive. Multiple low molecular weight peaks (Kav = 0.5–1.1) exhibited lower intrinsic activities (maximal secretory rate at maximum concentration of added product) than did oCRF in the in vitro bioassays and were not active in our CRF assay. We suspect that the smaller molecular weight bioactive substances with lower intrinsic activity than CRF may be related to neurohypoophysial peptides or catecholamines. Although these weak ACTH secretagogues may have limited independent effects, they may be important as potentiators of the action of CRF (9, 16–18, 29). The nature of the high molecular weight fraction (Kav = 0.0–0.15) has not been further investigated and may represent a precursor molecule (30). An estimate of the relative content in the rat hypothalamus of the high molecular weight versus the 4- to 5-kilodalton species may depend upon several factors, including detection methods, tissue collection, and extraction procedures. The zone representing 90% of the total CRF-like immunoreactivity detected after gel filtration was further purified batchwise by preparative HPLC with the Waters Associates preparative LC-500 system (step 1 in Table 1). Under these conditions rCRF and GH-RF activities could be clearly separated. Retention volume for rat GH-RF was 1,600–1,800 ml. A total of 100,000 rat hypothalami were processed in three batches (which we will refer to as batch numbers 1, 2, and 3, respectively) of 60,000, 20,000, and 20,000 hypothalami, which had a total dry weight of 500 g. Yields were 5.9, 1.6, and = 1.8 nanomol of rCRF, which had respective purities of 50–55%, 50–90%, and >90% as shown by sequence and amino acid analyses (31). Overall recovery was >50% as assessed by RIA of both starting materials and final product. This corresponds to an approximate 10^3-fold purification. Whereas we used batch 1 to optimize semipreparative and analytical chromatographic conditions that led to a preparation pure enough for sequence analysis and peptide mapping, batch 2 was used for confirmatory sequence analysis; batch 3 was purified, and an aliquot was further oxidized to [Met(O)]-rCRF in order to obtain an amino acid analysis that reflected the composition derived from sequence analysis and for chromatographic comparison with the synthetic requisite (Fig. 1). Details of the purification of batch 2 are outlined in Table 1.

A semipreparative step (1) on Vydac C4 was followed by five analytical steps on different packing materials (Vydac C4, diphenyl- or C18-derivatized 300-Å silicas) with two solvent systems (TEAP buffer or 0.1% CF3COOH and CH3CN). Step 7 was used to concentrate the earlier fractions from step 6 and free them from the 2-mercaptoethanol that had been added at each step to minimize oxidation of the methionine residue(s) prior to sequence analysis. Batch 3, which was purified following a strategy similar to that shown in Table 1, yielded before and after H2O2 treatment both rCRF and [Met(O)]-rCRF as illustrated in Fig. 1. In contrast to rCRF, which was isolated in its oxidized methionine form from a minor side fraction of extracts originally used for the characterization of lutheinizing hormone-releasing factor (20), rCRF was isolated in its native form and found to be the major ACTH-releasing substance with high intrinsic activity in fresh rat hypothalamic extracts.

Problems associated with the purification of hypophysiotopic peptides...
otrophic peptides from ovine or rat hypothalami have been well recognized (4, 32). We had observed that large-pore C18-derivatized silica (Vydac, 5-μm particle size, 300-Å pore size) gave significantly better resolution of CRF and, in general, of peptides larger than 25 residues than similarly derivatized silicas with pore sizes ranging from 80 to 120 Å (33). Usage of similar silicas with different selectivities obtained by derivatization and end-capping (C8, diphényl, and C18) and of two solvents (TEAP and 0.1% CF3COOH) with different ion-pairing capabilities has resulted in good resolution and excellent recovery of CRF biological and immunological activities.

The primary structure of rCRF was determined by Edman degradation using the α-phthalaldehyde strategy and peptide mapping on reverse-phase HPLC after clostripain digestion (31). The α-phthalaldehyde strategy consists of selectively block-

![Figure 1](image)

**Figure 1.** (A) To an aliquot of rCRF (1.200 hypothalami equivalent in 0.25 ml of 0.1% CF3COOH in 35% CH3CN/65% H2O) from step 7 in Table 1 diluted with water (0.25 ml) was added H2O2 (30%, 50 μl) at 0°C. The solution was kept at that temperature for 0.5 hr and was chromatographed under the conditions described below. The major peak (>90%) [Met(O)21,38]rCRF was collected for rchromatography in the presence of an equivalent amount of nonoxidized material to generate chromatogram A. (B) Synthetic [Met(O)21,38]rCRF was preparatively generated by treatment at 0°C of the synthetic rCRF (5 mg) in 1% AcOH (1 ml) with an excess of H2O2 (100 μl) for 0.5 hr. Aliquots (0.7 μg) of both synthetic peptides were rechromatographed to generate chromatogram B. Kinetic studies have shown that prior to generating [Met(O)21,38]rCRF, both intermediates [Met(O)21]rCRF and [Met(O)38]rCRF are formed and can be easily separated from both reduced and fully oxidized materials. The samples of native and synthetic peptides were applied in two successive runs to a Vydac C4 (no. 2115-6) end-capped (5-μm 300-Å pore size; 4.5 × 250 nm) column, which was eluted under gradient conditions shown by the dotted line. The solvent in A was 0.1% CF3COOH, the solvent in B was 0.1% CF3COOH in 80% CH3CN/40% H2O. Flow rate was 1.2 ml/min. UV detection at 210 nm is expressed in absorbance units full scale. Peaks A and C are native and synthetic [Met(O)21,38]rCRF; B and D are native and synthetic rCRF. a

A homology (83%) with oCRF is present (Fig. 2); substitutions are found at positions 2, 22, 23, 25, 38, 39, and 41. With the exception of the position 2 substitution, oCRF and rCRF shares a common 1-21 sequence, thus explaining the ability of the RIA with the C-24 antibody directed against the NH2-terminal 4-20 residues to read both CRFs well, whereas most RIAs developed against the whole oCRF sequence had been shown in several laboratories (23, 30) to only poorly detect CRF. RIAs with the C-24 antisera have been used to detect CRF-like immunoactivity in the hypothalamus/median eminence/pituitary of sheep, dog, rat, and human (9, 13, 14).

rCRF was synthesized in a stepwise manner on a paramethylenzhydrolyamine resin. The following sequence was assembled by using experimental procedures similar to those described earlier (32): Boc-Ser(OBzl)-Glu(OBzl)-Glu(OBzl)-Pro-Pro-Ile-Ser(OBzl)-Leu-Asp(OBzl)-Leu-Thr(OBzl)-Phe-His(Tos)-Leu-Leu-Arg(Tos)-Glu(OBzl)-Val-Leu-Glu(OBzl)-Met-Ala-Arg(Tos)-Ala-Glu(OBzl)-Gln(Xan)-Leu-Ala-Gln(Xan)-Gln(Xan)-Ala-His(Tos)-Ser(OBzl)-Asn(Xan)-Arg(Tos)-Lys(2CIZ)-Leu-Met-Glu(OBzl)-Ile-Ile-paramethylenzhydrolyamine resin. After complete deprotection and cleavage by hydrofluoric acid, the crude preparation of rCRF was purified by using preparative HPLC techniques developed in our laboratory (35). The synthetic peptide was shown to be greater than 98% pure by HPLC with several solvent systems and columns; it had the expected amino acid composition, and a peptide map after incubation with clostripain was generated by reverse-phase HPLC (31). No evidence for contaminating peptides could be shown. Native [Met(O)21,38]rCRF could be generated by H2O2 oxidation in 0.1% CF3COOH in the presence of 10-20% CH3CN. Synthetic [Met(O)21,38]rCRF was generated from synthetic rCRF after H2O2 oxidation in 1% AcOH as reported earlier for oCRF. As can be seen (Fig. 1), both natural and synthetic peptides cannot be distinguished from their chromatographic behavior in that system. Similarly, all other tests, including sequence analysis and peptide mapping of natural and synthetic rCRF, cannot show any significant difference.

The abilities of native rCRF, synthetic rCRF, and oCRF to stimulate ACTH secretion by cultured rat pituitary cells are compared in Fig. 3. In two assays, one of which is shown in Fig. 3, both synthetic rCRF and oCRF and native rCRF exhibit similar potencies and intrinsic activities. In the same assay, [Met(O)21,38]rCRF was found to have only 1% of CRF potency; hence justifying our efforts to keep our extracts in the presence of reducing agents (2-mercaptoethanol) throughout the purification scheme. In the rat, rCRF has been shown to be equipotent to oCRF and to exhibit similar activities (unpublished data).

We had shown that acetylated des-Ser-Gln-Glu-oCRF and des-Ser-Gln-Glu-Pro-Pro-oCRF had full intrinsic activity and 100% and 28% of CRF's potency, respectively, whereas oCRF (10-41) was almost devoid of biological activity, indicating that the NH2-terminal five or six residues were not indispensable for intrinsic activity (7). Thus, substitution of Gln-2 in oCRF by Glu-2 in rCRF would not have been expected to modify either the spectrum of activities or potency of rCRF. Substitutions at positions 22, 23, and 25 (and 38, 39, and 41), which are within a sequence stretch that was predicted [by using Chou and Fasman statistical analysis (36)] or proposed [by using circular dichroism spectroscopy and other physiochemical methods (T. Kaiser, M. Goodman, and P. Pullai, personal communication; unpublished data)] to have a highly amphiphilic α-helical sec-
ordinary structure encompassing residues 8–31 and possibly 7–41, could have had an important effect on biological activity. By using Chou and Fasman P values assigned to Thr-22 (0.83), Lys-23 (1.16), Asp-25,39 (1.01), Leu-38 (1.21), and Ala-41 (1.42) for oCRF and Ala-22 (1.42), Arg-23 (0.98), Glu-25,39 (1.51), Met-38 (1.45), and Ile-41 (1.02) for rCRF, we show a significant increase in a helix-forming potential. Furthermore, the character of the side chains has been conserved (Ala-22/Thr-22, Met-38/

Leu-38, and Ile-41/Ala-41, neutral; Arg-23/Lys-23, basic; Glu-25,39/Asp-25,39, acidic). The strengthening of the potential α-helical secondary structure of rCRF versus that of oCRF may further suggest that this structure is indeed the biologically active form that we proposed would interact with the receptor. Finally, all substitutions, with the exception of Ile-41 for Ala-41, may have resulted from single base changes.

Ovine CRF and human pancreatic tumor CRF (35) differ from the corresponding rat hypothalamic peptides by 17% and 33%, respectively. This marked nonalignment between mammalian regulatory peptides has not been seen for the smaller hypothalamic hypophysiotropic peptides.

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