Alanine Series of Ovine Corticotropin Releasing Factor (oCRF): A Structure–Activity Relationship Study†

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Previous structure–activity relationship studies of CRF have shown that residues 1–4 were not necessary for receptor binding or transduction, that residues 4–8 were important for activation, and that residues 12–41 were mostly responsible for binding. Finally it was proposed that CRF assumed an α-helical structure when interacting with its receptor. By systematic substitution of each residue (except residues 1–4) in ovine CRF (oCRF) by Ala, we have investigated the role played by individual side chains in receptor recognition and activation. Out of 33 analogues (synthesized using SPPS on an MBHA resin, purified by RPHPLC and characterized by amino acid and mass spectral analyses), a significant loss of biological potency (<1% potency of native) was observed for 6 analogues ([Ala^6], [Ala^8], [Ala^{10}], [Ala^{12}], [Ala^{14}], and [Ala^{38}]); 12 analogues had biological potencies ranging from 1% to 60% and ranked as follows: [Ala^{35}] < [Ala^{16}] < [Ala^9] < [Ala^{19}] < [Ala^{15}] < [Ala^{13}] < [Ala^7] < [Ala^{23}] < [Ala^{11}] ≤ [Ala^{21}] < [Ala^{27}] ≤ [Ala^{18}]; 8 analogues were found to be equipotent (>60% and <150%) ([Ala^5], [Ala^{17}], [Ala^{26}], [Ala^{29}], [Ala^{30}], [Ala^{34}], [Ala^{36}], and [Ala^{37}]; and 7 analogues were found to be approximately 2–5 times more potent than native oCRF ([Ala^{25}] = [Ala^{40}] ≤ [Ala^{39}] ≤ [Ala^{33}] < [Ala^{20}] < [Ala^{22}] < [Ala^{32}], in an in vitro pituitary cell culture assay. In summary, the Ala substitutions which showed the greatest loss of potency (<1% of native oCRF) were those replacing hydrophobic residues while those showing the greatest increase in potency were replacing hydrophilic residues. Of the 22 Ala-containing analogues in the C-terminal half of the molecule, 17 analogues have equal or greater potencies than native oCRF. Substitution of Ala in the N-terminal region (residues 5–19) on the other hand is generally detrimental to biological activity. These results suggest that the side chains of residues 5–19 are very important for receptor binding and activation while, in the C-terminal region, the amino acid side chains may be more responsible for structural conservation than for functional expression.

Introduction

The mechanisms that modulate the release of adrenocorticotropic hormone (ACTH) by the pituitary involve the interaction of several secretagogues which include corticotropin releasing factor (CRF), arginine-vasopressin, catecholamines, and angiotensin-II (for review, see Rivier et al., 1990[1]). Corticotropin releasing factor, a 41 amino acid peptide first isolated from sheep (oCRF)[2], a relative of sauvagine[3,4] and the urotensins, and later characterized in a variety of species[5–19] (Table I), is the principal neuroregulator of ACTH secretion. The secretion of β-endorphin and other proopiomelanocortin products of the anterior pituitary gland are also regulated by CRF.[2,20–22] In the central nervous system (CNS) CRF has been shown to be distributed broadly[23] and to exert a wide spectrum of CNS-mediated effects on behavior,[24,25] the cardiovascular system,[26,27] reproduction,[28,30] gastrointestinal secretion,[31,32] motility,[33] and transit.[34] This suggests that within the brain this peptide plays an important role in the etiology of stress.[35,36] The underlying brain mechanisms involved in stress-induced behavioral, metabolic, and endocrine functions are still poorly understood. Yet, the availability of competitive antagonists of CRF was critical in defining the potential role played by CRF on the effects of stress.[28,32,34–37] Because it is believed that potent and safe CRF antagonists could play a major role in the understanding and possible management of some stress-related pathological states, we[38–42] and others[43,44] have investigated CRF's structural basis for biological action which is still poorly understood. We already know (from the fact that the C-terminal fragment of CRF is a competitive antagonist) that the first eight residues of the CRF molecule are involved in receptor activation and that the remaining C-terminus may be responsible for significant binding affinity to the receptor.[38]

The existence of multiple forms of CRF receptor subtypes has been demonstrated[37,45] by comparing the ability of the CRF antagonist, α-helical CRF(9–41), to inhibit the actions of CRF in three in vivo bioassay systems which evaluated the pituitary, the cardiovascular, and central

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Table I. The CRF, Sauvagine, and Urotensin Family

	1 5 10 15 20 25 30 35 40
Ovine/Caprine CRF[2,3]	S Q E P P I S L D L T F H L L R E V L E M T K A D Q L A Q Q A H S N R K L L D I A-NH_2
Bovine CRF[2]	- N - - - - - - - --NH_2
Human/Rat CRF[7,9]	- E - - - - - - - - - - - - - - - - - - - A R - E - - - - - - - - - - - - - M E - I-NH_2
Porcine CRF[15]	- E - - - - - - - - - - - - - - - - - - - A R - E - - - - - - - - - - - - - M E N F-NH_2
Sucker CRF[17]	- E - - - - - - - - - - - - - - - - - - - A R - E - - - - - - - - - - - - - M M E - F-NH_2
Frog Sauvagine[3,4]	<- G - - - - - - - S L E - - - K M I - I E - Q E K E K - - - A N - - L - - - - T I-NH_2
Sucker Urotensin I[6,11]	N D D - - - - I - - - - - - - - - N M I - - A R I E N E R E - - G L - - - Y - - E V-NH_2
Carp Urotensin I[5]	N D D - - - - I - - - - - - - - - N M I - - A R N E N Q R E - - G L - - - Y - - E V-NH_2
Sole Urotensin I[16]	- E - - - M - I - - - - - M - - N M I H R A - M E G E R E - - L I - - N - M - E V-NH_2
Maggy Urotensin I[6]	- E - - - M - I - - - - - M - - N M I H R A - M E G E R E - - L I - - N - - - E V-NH_2
Flounder Urotensin I[19]	- E D - - M - I - - - - - M - - N M I H - A - M E G E R E - - Q I - - N - - - E V-NH_2

nervous systems. The demonstration of variable potency for α-helical CRF(9–41) on inhibition of CRF-induced activity, at CNS and vascular receptors, supports the existence of multiple CRF receptor subtypes. Further evi-

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dence demonstrated that the brain receptor for CRF has a lower apparent molecular weight than the CRF receptor in the anterior pituitary.[46] The difference that was observed in the molecular weights for the brain and pituitary CRF receptors may in fact be due to glycosylation only, or to the fact that the authors may have been looking at the CRF binding protein.

Introduction of structural modifications into native peptides allows the determination of the amino acid residues which play a functional role in the secondary structure of the peptide and in binding of the peptide to its receptor by demonstration of the retention of biological activity or potency. Residues or regions of the peptide where future manipulations might be focused can be derived from studies on backbone conformational changes and through selective amino acid substitutions. Biological and structural results (backbone conformation) derived from the systematic substitution of each residue in oCRF by its D-isomer are described elsewhere.[40] Similarly, N^{α} and C^{α} substitutions of selected residues in CRF (as well as some circular dichroism studies) were carried out (Hernandez et al., in preparation) and confirmed earlier studies[38] which suggested that CRF may assume, at least in part, an α-helical secondary structure when interacting with its receptor.[9,38,41,42]

In order to develop analogues of oCRF with different selectivities and therefore greater affinity for the different receptors recognized so far, it is necessary to determine which structural and conformational parameters are important for biological potency. Because we have derived from the study of the D-amino acid substitutions an understanding of the role played by individual amino acids in allowing a bioactive backbone conformation, we are now addressing the role played by individual side chains in the recognition and activation process by substituting Ala sequentially for each amino acid in oCRF. Alanine is the smallest amino acid which possesses an optically active α-carbon and a nonfunctional, hydrophobic side chain which is assumed to be a moderate α-helical promoter. The ability of the resultant oCRF analogues to induce the secretion of ACTH from anterior pituitary cells and the relative potencies of these analogues are discussed with respect to the suspected role of individual side chains in receptor binding and activation.

Results

Synthesis, Purification, and Characterization (Table II). Peptide analogues were synthesized using solid-phase methodology, either manually or automatically (using a program developed in house) on a Beckman Model 990 peptide synthesizer, on *p*-methylbenzhydrylamine resin (MBHA resin) by use of previously described protocols.[47] Briefly, *tert*-butoxycarbonyl (Boc) was used for N-terminal protection with N-terminal deblocking performed with TFA–CH_2Cl_2 (3:2) in the presence of 1% ethanedithiol (EDT) twice for 15 min. Coupling was mediated using 1,3-diisopropylcarbodiimide (DIC) or dicyclohexylcarbodiimide (DCC) in CH_2Cl_2 (DCM) or 50% DCM–dimethylformamide (DMF), depending on the solubility of the Boc-amino acids. Difficult couplings were accomplished using 1-hydroxybenzotriazole (HOBt) or HOBt and (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP, 1:1) in DCM or 50% DMF–DCM in the presence of excess diisopropylethylamine. Coupling time was for 90–120 min, with recouplings done where needed. Washes included 2-propanol (containing 1% EDT), DCM, and methanol (MeOH). Triethylamine (TEA) (10% in DCM) was used for neutralization of the peptide resin after TFA deblocking. The protected peptide resin was cleaved in anhydrous HF in the presence of 3% anisole and 3% methyl sulfide at 0 °C for 90 min. The HF was then removed in vacuo. The crude peptides were precipitated with anhydrous diethyl ether, separated from ether-soluble nonpeptide material by filtration, extracted from the resin with 30% acetonitrile (MeCN) in water, and lyophilized.

Crude peptides were purified by preparative reverse-phase HPLC (RPHPLC) usually in three steps. The crude lyophilized peptides (1–2 g) were dissolved in MeCN–water (30:70) (2–300 mL) and loaded on a preparative reversed-phase HPLC cartridge (5- × 30-cm) packed in house using Vydac C_{18} silica gel (The Separations Group, Hesperia, CA; 300-Å pore size, 15–20-μm particle size).[48] The peptides were eluted using a linear gradient (40–70% B in 60 min) containing a mixture of solvents A (TEAP, pH 2.25) and B (60% MeCN in A) at a flow rate of 100 mL/min. Individual fractions (50–150 mL) were collected and analyzed by HPLC on a Vydac C_{18} silica gel column (0.46- × 25-cm, 5-μm particle size, 30-nm pore size) under isocratic conditions such that the elution time was greater than 4 min. Selected fractions were collected and diluted (1:1) with water and loaded on a preparative cartridge as above and eluted using a linear gradient (40–70% B in 60 min) containing a mixture of solvents A (TEAP, pH 5.30

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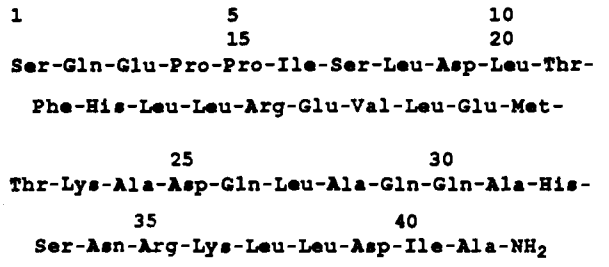
Table II. HPLC Data and Biological Activity for the Ala Series of oCRF



[Ala^{xx}]oCRF-substituted analogue	HPLC data[a] 0.1% TFA purity, %	0.1% TFA RT, min	TEAP 2.25 purity, %	TEAP 2.25 RT, min	relative in vitro potency[b] (95% confidence limits)
oCRF					1.00 (standard)
[Ala^{5}]oCRF	99.0%	16.4	99.5%	16.5	0.6 (0.35–1.0)
[Ala^{6}]oCRF	97.4%	14.0	97.9%	14.2	0.004 (0.001–0.009)
[Ala^{7}]oCRF	96.0%	20.6	96.8%	20.3	0.16 (0.08–0.34)
					0.21 (0.10–0.40)
[Ala^{8}]oCRF	96.0%	12.7	95.5%	12.7	0.003 (0.001–0.006)
					0.005 (0.003–0.008)
[Ala^{9}]oCRF	94.7%	17.1	93.5%	16.7	0.04 (0.020–0.08)
[Ala^{10}]oCRF	97.0%	14.5	95.3%	15.2	0.006 (0.003–0.010)
[Ala^{11}]oCRF	98.4%	19.7	98.8%	20.0	0.4 (0.2–0.8)
[Ala^{12}]oCRF	99.6%	13.7	97.6%	13.6	0.006 (0.002–0.014)
[Ala^{13}]oCRF	97.5%	18.7	97.2%	19.0	0.14 (0.08–0.28)
[Ala^{14}]oCRF	89.2%	12.4	95.4%	12.3	0.001 (0.00–0.002)
					0.001 (0.00–0.001)
[Ala^{15}]oCRF	97.3%	12.2	90.1%	12.1	0.09 (0.03–0.2)
[Ala^{16}]oCRF	94.5%	18.3	95.7%	18.7	0.02 (0.01–0.04)
[Ala^{17}]oCRF	99.3%	17.8	98.9%	17.5	0.6 (0.2–2.4)
[Ala^{18}]oCRF	99.6%	13.7	99.5%	13.8	0.5 (0.2–1.4)
[Ala^{19}]oCRF	97.5%	12.8	99.5%	12.7	0.09 (0.03–0.25)
[Ala^{20}]oCRF	98.0%	17.0	96.0%	16.2	2.8 (1.2–4.9)
					1.6 (0.8–2.9)
[Ala^{21}]oCRF	99.8%	14.9	99.9%	15.1	0.4 (0.2–0.7)
[Ala^{22}]oCRF	99.0%	17.5	99.0%	17.2	3.4 (1.7–6.7)
[Ala^{23}]oCRF	96.0%	19.0	98.0%	19.5	0.28 (0.07–1.0)
[Ala^{24}]oCRF	native	native	native	native	native
[Ala^{25}]oCRF	99.0%	18.2	99.5%	17.8	1.7 (0.9–3.1)
[Ala^{26}]oCRF	95.4%	18.2	96.0%	18.1	1.1 (0.5–2.3)
[Ala^{27}]oCRF	97.3%	15.6	98.5%	15.7	0.6 (0.16–2.1)
					0.38 (0.14–1.0)
[Ala^{28}]oCRF	native	native	native	native	native
[Ala^{29}]oCRF	98.0%	16.2	99.4%	17.4	1.0 (0.6–1.6)
					1.4 (0.7–3.0)
[Ala^{30}]oCRF	98.0%	17.5	97.0%	17.1	0.8 (0.4–1.6)
[Ala^{31}]oCRF	native	native	native	native	native
[Ala^{32}]oCRF	96.4%	18.5	95.4%	18.7	4.5 (2.8–7.1)
[Ala^{33}]oCRF	99.5%	17.3	99.6%	16.8	2.0 (1.1–3.4)
[Ala^{34}]oCRF	96.1%	17.8	98.2%	18.4	1.1 (0.7–1.7)
[Ala^{35}]oCRF	78.1%	18.2	84.9%	19.4	0.02 (0.006–0.06)
[Ala^{36}]oCRF	98.0%	18.5	97.8%	19.5	0.8 (0.4–1.4)
[Ala^{37}]oCRF	89.0%	16.3	89.2%	16.6	0.7 (0.3–1.5)
[Ala^{38}]oCRF	97.9%	16.2	99.1%	16.5	0.004 (0.001–0.008)
					0.0002 (0.000–0.001)
[Ala^{39}]oCRF	91.6%	16.6	92.5%	17.4	1.8 (1.0–3.5)
[Ala^{40}]oCRF	97.6%	15.1	98.0%	16.5	1.7 (1.0–3.2)
[Ala^{41}]oCRF	native	native	native	native	native

[a] RT = retention time. HPLC analysis was performed in two mobile-phase systems, (1) 0.1% TFA–MeCN and (2) TEAP (pH 2.25)–MeCN, on a Vydac C_{18} silica gel column (0.46 × 25-cm, 5-μm particle size, 30-nm pore size) with UV detection at 210 μm and a flow rate of 1.5 mL/min. [A] = 0.1% TFA or TEAP 2.25, and [B] = 0.1% TFA in 60% MeCN or 60% MeCN in [A] (TEAP 2.25). Gradients used were (1) for the 0.1% TFA system = 55% [B] to 85% [B] in 30 min and (2) for the TEAP 2.25 system = 50% [B] to 80% [B] in 30 min. [b] Potencies were determined from parallel dose–response curves versus oCRF of single bioassays and calculated using the BIOPROG program.[52] Two entries indicate that the peptide has been tested twice.

to pH 6.50) and B (60% MeCN in A) at a flow rate of 100 mL/min. Individual fractions were analyzed as above under isocratic conditions and selected fractions diluted (1:1) with water. These fractions were converted to the TFA salt by loading on a preparative cartridge as above and eluted using a linear gradient (45–75% B in 20 min) containing a mixture of solvents A (0.1% TFA) and B (60% MeCN, 0.1% TFA) at a flow rate of 100 mL/min. Selected fractions were lyophilized. After lyophilization we obtained relatively large quantities (50–100 mg) of highly purified peptides.

Purified peptides were subjected to HPLC analysis in two mobile-phase systems, 0.1% TFA–MeCN and TEAP (pH 2.25)–MeCN, on a Vydac C_{18} silica gel column (0.46- × 25-cm, 5-μm particle size, 30-nm pore size). Purity of the peptides, synthesized over a 9-year period, were found to be generally greater than 95% (Table II). The peptides were subjected to further characterization using the ana-

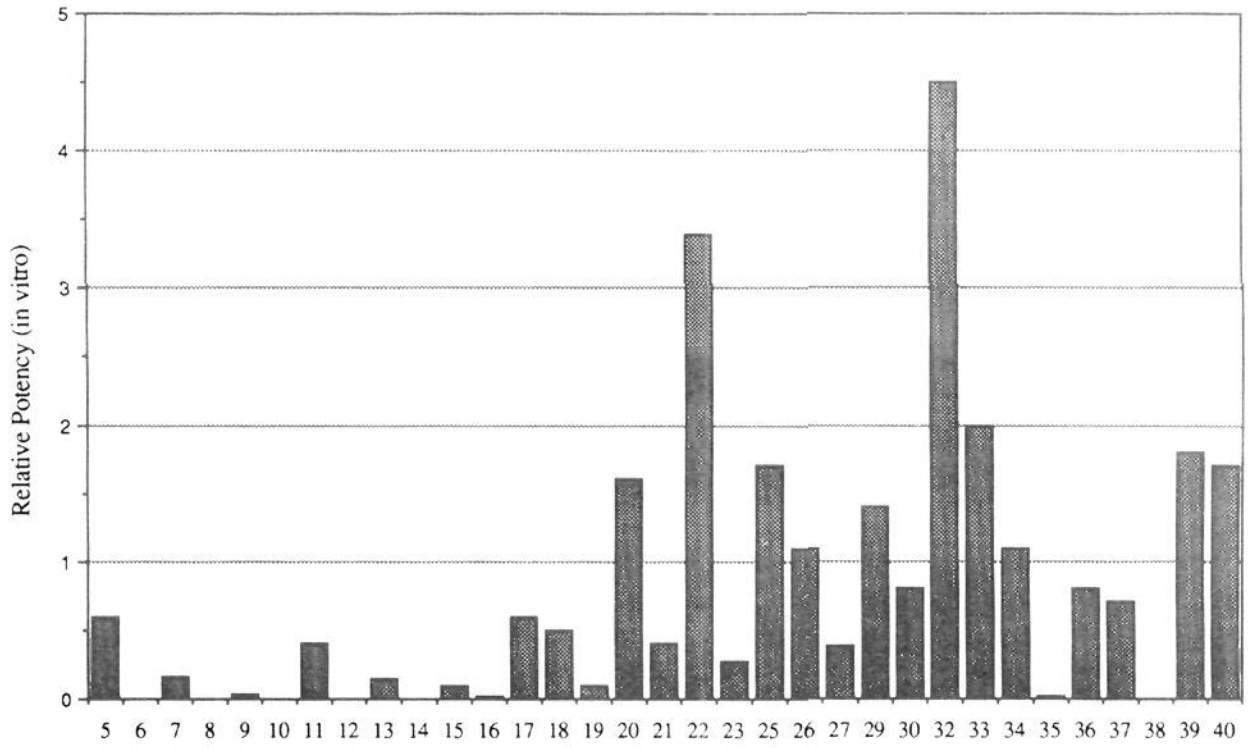


Figure 1. Graphic representation of biological data presented in Table II.

lytical techniques of amino acid analysis, mass spectrometry, optical rotation, and determination of in vitro relative potency (see Table II). Amino acid and mass spectrometric analysis gave results which were consistent with the expected structures. The optical rotations of the Ala-substituted analogues were not greatly influenced by the introduction of Ala at any of the positions substituted in this study. The lack of influence of a single amino acid substitution on optical rotation was also found in the D-amino acid series.[40]

Biological Activity (Table II, Figure 1). The ability of the peptides to stimulate ACTH release by rat anterior pituitary cells was determined in vitro.[22] This in vitro assay is similar to the one used for the isolation and characterization of oCRF.[2] The potency of each analogue is reported relative to that of synthetic oCRF.

Discussion

In order to understand which structural parameters are important for binding and activation, we have first identified the smallest fragment [CRF(5–41)] with CRF-like activity and comparable potency.[9,38] Then we have (a) identified CRFs binding (residues 9–41) and activating (residues 4–8) regions,[9,38] (b) determined that the binding region would likely assume an α-helical structure when interacting with its receptor,[9,38] and (c) shown that introduction of D residues in certain positions (residues 12 and 20) yielded analogues with high potency while all other members of the D series (except residues 1–4 which were not synthesized) were either partially active or impotent.[39,40]

Here, we have addressed the question of the role of individual amino acid side chains on biopotency by scanning the CRF molecule with individual Ala residues. On the basis of this scan we have identified compounds that were either inactive (or with very low potency), had a potency ranging from 1% to <60% that of oCRF, were equipotent (>60% to 150%), or were more potent than that of oCRF (>150%). In the cases where the same analogue was tested twice (see Table II), the results consistently show good concordance between relative potencies, thus validating the assay further. Of greatest interest to us were those analogues that had residual biopotency (<1%) because of the fact that they may be competitive antagonists. When tested for antagonistic activity, none of these analogues were active. These were analogues in which Ala replaced [Ile^6], [Leu^8], [Leu^{10}], [Phe^{12}], [Leu^{14}], and [Leu^{38}]. Interestingly, all of these residues contain hydrophobic side chains which are mostly conserved (Ile goes to Met, Leu goes to either Ile or Met, and Phe goes to Leu) among the members of the CRF family. Whether these residues play a crucial role in stabilizing the secondary structure of CRF when interacting with the receptor or are individually responsible for a direct interaction with the receptor is still to be determined. A third possibility would be that those residues are involved in stabilizing a quaternary structure encompassing more than one CRF unit. All these hypotheses are presently being investigated.

A significant loss of biological potency (1% to <60% that of oCRF) was observed for 12 analogues in which Ala replaced [Ser^7], [Asp^9], [Thr^{11}], [His^{13}], [Leu^{15}], [Arg^{16}], [Val^{18}], [Leu^{19}], [Met^{21}], [Lys^{23}], [Leu^{27}], and [Arg^{35}]. Interestingly, five of these residues ([Ser^7], [Asp^9], [Leu^{15}], [Arg^{16}], and [Arg^{35}]) are completely conserved throughout all the presently known sequences of the members of the CRF family (Table I). Other residues are functionally conserved ([Thr^{11}], [Val^{18}], [Leu^{19}], and [Lys^{23}]). Functionality refers to chemical properties of the side-chain groups of the amino acids (i.e. hydrophobic versus hydrophilic, neutral versus charged, acidic versus basic, etc.). Finally, three residues ([His^{13}], [Met^{21}], and [Leu^{27}]) are completely conserved within the CRFs, but in sauvagine these residues are not conserved, with functionality being conserved for [Met^{21}] and not for [His^{13}] and [Leu^{27}]. In the urotensin family [His^{13}] was completely conserved and [Met^{21}] was partially conserved structurally and/or functionally, while [Leu^{27}] was not conserved either structurally or functionally. All of these analogues had full intrinsic activity. These results suggest that the side chains at these positions are important, yet not as critical for the binding and activation processes as those side chains identified in the previous series of substitutions. Eight analogues of oCRF in which Ala-substituted [Pro^5], [Glu^{17}], [Gln^{26}], [Gln^{29}], [Gln^{30}], [Asn^{34}], [Lys^{36}], and [Leu^{37}] were shown to be equipotent (>60% to 150%) with oCRF. The positions identified here are either completely conserved ([Pro^5], [Gln^{30}], and [Asn^{34}]) or conserved ([Glu^{17}], [Gln^{26}], [Gln^{29}], [Lys^{36}], and [Leu^{37}]) within the CRF family but not in sauvagine and/or within the urotensin family. Interpretation of this data is more difficult in the absence of a strong conformational or functional hypothesis. Finally, an increase in biological activity from 2–5-fold over that of native oCRF was observed for seven analogues in

which Ala replaced [Glu^{20}], [Thr^{22}], [Asp^{25}], [His^{32}], [Ser^{33}], [Asp^{39}], and [Ile^{40}]; none of the substitutions in these analogues are completely conserved. In two cases, [Asp^{25}] and [Asp^{39}], functionality is conserved since the Asp residues are found as Glu residues in either the urotensins, CRFs, or in sauvagine. In all other cases, functionalities were not conserved which suggested that those residues in the different species may have evolved independently along with their respective receptors to fulfill independent functions. The CRFs, urotensins and sauvagine are about equipotent in inducing the release of ACTH from the pituitary.[49,50] However, the CRFs are 5–10 times less potent than sauvagine and the urotensins when acting within the brain to increase the plasma levels of catecholamines and glucose and in elevating mean arterial pressure.[50,51] Sauvagine is also more potent than the CRFs when acting outside the brain to increase superior mesenteric artery flow and plasma glucose concentrations and to decrease mean arterial pressure.[50]

Of the four Ala residues found in oCRF (positions 24, 28, 31, and 41) one should note that only [Ala^{31}] is fully conserved. At the other three positions one can find Gln, Ile, Asn, Met, Arg, Lys, Phe, or Val in the other known members of the CRF family (Table I).

While all members of the CRF family show equal potency to release ACTH in a mammalian in vitro system, those members isolated from mammalian and fish (sucker) brains have sequences that are considerably more conserved than that of the members isolated from the urophysis of different fish or the skin of the frog *Phyllomedusa sauvagei*. Of interest is the fact that most sequence differences between the brain CRFs and urophysins are located between residues 17 to 29 where single alanine substitution has the least effect on biopotency with the exception of [Ala^{19}].

An interesting observation is that all of the Ala-substituted analogues that were found to be of equal or higher potency than native oCRF were found in the C-terminal region (from position 20 through 41). Thus, of the 22 Ala-containing analogues in this C-terminal region, 17 analogues, or 77%, have equal or greater potency than native oCRF. Substitution of Ala in the N-terminal region (residues 5–19) was always detrimental to biological activity. These results suggest that the side chains of residues 5–19 are very important for receptor binding and biological activity. While in the C-terminal region, the amino acid side chains may be more responsible for structural conservation than for functional expression. Consistent with this observation is the fact that the amino acids in the N-terminal region of the members of the CRF family are more highly conserved than in their C-terminal region. Another observation was that all substitutions of either glutamic acid, aspartic acid, or glutamine by Ala were found to yield compounds with equal (positions 26 and 30) or greater potencies (positions 20, 25, 29, and 39) than native oCRF, except the substitutions of Ala for [Asp^{9}] and [Glu^{17}], which are found in what we have defined as the N-terminal region.

While the above deductions result from direct analysis of primary structure–activity relationships, we can now speculate about the secondary structure–activity relationships with the understanding that most reports describing the secondary structure of the members of the CRF family are limited, being either the results of statistically based predictions or CD spectroscopic data that lack the precision of NMR or crystallographic data. Ovine CRF, sauvagine, and sucker urotensin I contain many secondary structural similarities and some notable differences.[41–43] The greatest structural differences are proposed to be in the N-terminal region (residues 1–7) and in the C-terminal region (residues 36–41) while most structural similarities are proposed to be between residues 8 and 35. β-Turns are prediced to be in the N-terminal region (approximately residues 1–5) and around residues 31–35 for oCRF, sauvagine, and sucker urotensin I.[42,43] Sauvagine is also predicted to have a β-turn around residues 5–9. All contain a long internal segment of about 25 amino acids (around residues 8–31) that is predicted to be α-helical in nature. Sauvagine is the only peptide predicted to contain a β-sheet, which is in the C-terminal region (residues 36–40). Since Ala is supposed to be α-helical-promoting it may be expected that substituting Ala in the α-helical region would have very little effect on potency, except for the residues that are directly involved in binding or biological activity. We found that Ala substitution had the greatest effect on loss of biological activity in the N-terminal region (residues 5–19) and the least effect in the C-terminal region (residues 20–41). These results suggest that residues 5–19 are more important for biological activity than residues 20–41, which might be more involved in the secondary structure of CRF. However, the entire C-terminal region in CRF is required for full potency since CRF COOH-terminal-free acid and CRF(1–39)-NH_2 have less than 0.1% of the potency of oCRF(1–41)-NH_2.[2] This would agree with the overall sequence homology of the CRF, sauvagine, and urotensin families, which share greater sequence homology in the N-terminal region than in the C-terminal region.

In summary, oCRF analogues that are monosubstituted by Ala have been synthesized and found to be from 4.5 times more potent to <0.001% as potent as native oCRF. The Ala substitutions which showed the greatest loss of potency (<1% of oCRF) were replacing hydrophobic residues ([Ile^{6}], [Leu^{8}], [Leu^{14}], and [Leu^{38}]), while the Ala analogues which showed the greatest increase in potency were replacing hydrophilic residues ([Thr^{22}] and [His^{32}]). While such systematic studies lack the luster of hypothesis-directed research, it is still the most efficient approach to identify which residues within the sequence of a biologically active peptide are most sensitive to alteration. The next and yet unproven step is to identify whether some or all of the recognized, beneficial substitutions can be introduced in the same molecule and be additive in generating potent analogues. Since deletion of the first 12 residues will generate peptides which are competitive antagonists and all Ala substitutions that yielded more potent analogues than oCRF are found to be beyond residue 19, it is likely that potent CRF antagonists (expected to play an important therapeutic role in medicine) will incorporate selected Ala substitutions.

Experimental Section

All reagents and solvents were analytical grade and were used without further purification, except TFA and TEA, which were

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reagent grade and used without further purification for peptide synthesis and were distilled to constant boiling point for further use in the preparation of chromatographic buffers.

Instruments. Analytical HPLC analysis was done on a system composed of an Altex 420 system controller, two Altex model 100A pumps, a Rheodyne 7125 injector, a Vydac C_{18} column (0.46- × 25-cm, 5-μm particle size, 30-nm pore size), a Kratos Spectroflow 757 UV detector (detection was at 210 nm), a Houston Instruments OmniScribe Series D500 dual-pen chart recorder, and a Shimadzu Chromatopac E1A integrator or on a system composed of a Waters Associates automated gradient controller, two Waters M-45 pumps, a Kratos Model SF 769Z UV detector, a Rheodyne 7125 injector, a Shimadzu Chromatopac E1A integrator, and a Houston Instruments OmniScribe dual-pen chart recorder. Optical rotations were determined with a Perkin-Elmer Model 241 polarimeter in 1% acetic acid (c = 1.0) at room temperature (~23 °C) using a 100-μL cell.

Peptide Synthesis. Methylbenzhydrylamine resin (MBHA resin) (2–3 g per peptide) was prepared in house by the method of Rivier et al.[47] from polystyrene cross-linked with 1% divinylbenzene (Biobeads SX-1, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA). Resins with substitutions varying from 0.3 to 0.65 mequiv/g were used with no observable differences. Boc-Amino acids were purchased from Bachem (Torrance, CA). Threefold-excess protected Boc-amino acid was used on the basis of the original substitution of the MBHA resin. The reagents DIC, HOBt, TEA, anisole, methyl sulfide, acetic anhydride, and diisopropylethyl amine were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI); DMF, acetonitrile (reagent grade and ChromAR HPLC grade), and 2-propanol were purchased from Mallinckrodt Inc. (Paris, KY); MeOH and DCM were purchased from Fisher Scientific Co. (Fair Lawn, NJ); DCC and EDT were purchased from Fluka Chemie AG (Switzerland); BOP was purchased from Richelieu Biotechnologies, Inc. (Quebec, Canada); TFA was purchased from Halocarbon Products Corp. (Hackensack, NJ).

Peptide Purification. Preparative HPLC purification of the crude peptides was performed on a system consisting of either a modified Waters Associate (Milford, MA) 500A Prep LC, a Waters preparative gradient mixer or Eldex Chromatrol II gradient former, a Waters 450 variable-wavelength detector (detection was at 230 nm), and a Houston Instruments OmniScribe chart recorder or on a system composed of a Waters Associates Prep LC 3000 System, a Waters Associate 600E system controller, a Shimadzu SPD-6A UV spectrophotometric variable-wavelength detector, and a Fisher (Lexington, MA) Recordall Series 5000 stripchart recorder. The cartridges used were hand-packed, in house, with Waters polyethylene sleeves and frits and Vydac bulk C_{18} material (15–20-μm particle size, 30-nm pore size). Analytical HPLC analysis was performed on a Waters Associates HPLC system which was comprised of two M-45 pumps, a Waters Associates WISP sample injector, a Kratos Spectroflow model 773 UV detector, and a Waters Associates data module integrator/recorder.[48]

Amino Acid Analysis. Amino acid analysis of the peptides, following hydrolysis in 4 N methanesulfonic acid at 110 °C for 24 h, was performed on a Perkin-Elmer LC system comprised of two Series 10 LC pumps, a ISS-100 sample injector, a RTC 1 column oven, a Kratos Spectroflow 980 fluorescence detector, and a LCI-100 integrator. A Pierce AA511 ion exchange column was maintained at 60 °C and post column derivatization with o-phthaldehyde was performed at 52 °C. Samples containing the internal standard γ-aminobutyric acid were injected and 5 min after injection were subjected to a gradient of 0 to 100% B in 25 min and then 100% B for 15 min with a flow rate of 0.5 mL/min. Buffers A and B are Pierce Pico buffer (pH 2.20) and Beckman Microcolumn sodium citrate buffer (pH 4.95), respectively.

Optical Rotations. Optical rotations were measured in 1% acetic acid (c = 1.0; i.e. 10 mg of lyophilized peptide/mL, uncorrected for TFA counterions or water present after lyophilization). Values were obtained from the means of 10 successive 5-s integrations determined at room temperature (~23 °C) on a Perkin-Elmer 241 polarimeter (using the D line of Na emission) and are quoted as uncorrected specific rotations.

LSIMS. Mass spectra were measured using a JEOL JMS-HX110 double focusing mass spectrometer (JEOL, Tokyo, Japan) fitted with a Cs^+ gun. An accelerating voltage of 10 kV and Cs^+ gun voltage of 25 kV were employed. The sample was added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix.

Biological Testing. Rat anterior pituitary cells were prepared as previously described[22] by separation of the anterior lobe (rostral and proximal partes distales) from the posterior (neurointermediate) lobe, which was discarded. The anterior lobes were dispersed with collagenase for 1.5–2 h on a magnetic stirrer and established for 3–5 days in culture in β-PJ medium which contained 2% fetal calf serum. For the secretion experiments, the cells were washed by multiple media change using β-PJ medium containing 0.1% bovine serum albumin (BSA). After preincubation for 1 h in the above buffer, fresh media was applied and the cells were treated for 3 h with the test peptide. The level of release of ACTH in the incubation media was measured by radioimmunoassay (using radioimmunoassay kits from Diagnostic Products Corporation). Relative potencies[52] are shown (with respect to native oCRF), with 95% confidence limits in parentheses in Table II. A unique value was reported in the figure for the reader's convenience.

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