Minimal-Size, Constrained Corticotropin-Releasing Factor Agonists with *i*−(*i*+3) Glu−Lys and Lys−Glu Bridges†

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In three earlier publications (Miranda et al. *J. Med. Chem.* **1994**, *37*, 1450−1459; **1997**, *40*, 3651−3658; Gulyas et al. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10575−10579) we have hypothesized that covalent constraints such as side-chain-to-side-chain lactam rings would stabilize an α-helical conformation shown to be important for the recognition and binding of the CRF C-terminus 30 residues, to CRF receptors. These studies led to the discovery of useful CRF antagonists such as α-helical CRF (α-hel-CRF) and Astressin both in vitro and in vivo. To test the hypothesis that such lactam rings may also be modulating activation of the receptor when introduced at the N-terminus of CRF, we studied the influence of the successive introduction from residues 4 to 14 of a cyclo(*i*,*i*+3)[Lys^{i}−Glu$^{(i+3)}$] and a cyclo(*i*,*i*+3)[Glu^{i}−Lys$^{(i+3)}$] bridge on the in vitro potency of the agonist [Ac-Pro^{4},D-Phe^{12},Nle^{21,38}]hCRF$_{(4-41)}$ and related compounds. We have also introduced the favored cyclo(Glu^{30}−Lys^{33}) substitution found to be remarkable in several families of antagonists (such as Astressin) and in a number of CRF agonists and investigated the role of residues 4−8 on receptor activation using successive deletions. Earlier studies had shown that in both oCRF and α-helical CRF, deletion of residues 1−6, 1−7, and 1−8 led to gradual loss of intrinsic activity (IA) (from 50% IA to <10% IA) resulting in α-hel-CRF being a potent competitive antagonist. We show that acetylation of the N-terminus of these fragments generally increases potency by a factor of 2−3 with no influence on IA. While cyclo(30−33)[Ac-Leu^{8},D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(8-41)}$ (**30**) is the shortest reported analogue of CRF to be equipotent to CRF (70% IA), the corresponding linear analogue (**31**) is 120 times less potent (59% IA). Addition of one amino acid at the N-terminus {cyclo(30−33)[Ac-Ser^{7},D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(7-41)}$ (**28**)} results in a 5-fold increase in agonist potency and full intrinsic activity (113%). The most favored modifications were also introduced in other members of the CRF family including sauvagine (Sau), urotensin (Utn), urocortin (Ucn), and α-hel-CRF. Parallel and consistent results were obtained suggesting that the lactam cyclization at residues 29−32 and 30−33 (for the members of the CRF family with 40 and 41 amino acid residues, respectively) will induce (in the shortened agonists) a structural constraint (α-helix) that stabilizes a bioactive conformation similar to that shown in the Astressin family of CRF antagonists and that residue 8 (leucine or isoleucine) bears the sole responsibility for activation of the receptor since deletion of that residue leads to potent antagonists (Gulyas et al. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 10575−10579).

Introduction

Adequate functioning of the central nervous system leading to maintenance or restoration of homeostasis depends on the appropriate balance between a vast array of stimulatory and inhibitory neurotransmitters that counterbalance the effects of stressful stimuli. Critical in the maintenance of homeostasis is corticotropin-releasing factor (CRF), a peptide first isolated and characterized from sheep hypothalami.[4] We found that CRF plays an essential role in regulating the activity of the hypothalamic−pituitary−adrenal (HPA) axis.[5] Through the release of glucocorticoids, CRF also alters immune parameters[6] and participates in the regulation of carbohydrate metabolism by enhancing the availability of glucose (reviewed in ref 7). CRF was also subsequently found in extrahypothalamic regions, including the forebrain, the limbic system, and the brainstem, where it regulates behavior and vegetative functions including cardiovascular responses. CRF is considered to stimulate many of the functions that help the organism survive (such as locomotor activity and catecholamine release) while inhibiting those that might interfere with an effective stress response (such as

† IUPAC rules are used for nomenclature of peptides including one-letter codes for amino acids. Abbreviations: Ac, acetyl; ACTH, adrenocorticotropin hormone; Astressin, cyclo(30−33)[D-Phe^{12},Nle^{21,38},Glu^{30},-Lys^{33}]hCRF$_{(12-41)}$; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazolyloxy)tris(dimethylamino)phosphonium hexafluorophosphate; cHex, cyclohexyl; CRF, corticotropin-releasing factor (o, ovine; h, human); CZE, capillary zone electrophoresis; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HF, hydrogen fluoride; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; IA, intrinsic activity; MBHA, methylbenzhydrylamine resin; NMP, *N*-methylpyrrolidone; OFm, *O*-fluorenylmethyl; PTH, parathyroid hormone; Sau, sauvagine; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TEAP 2.25, triethylammonium phosphate, pH 2.25; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Ucn, urocortin; sUtn, sucker urotensin; Xan, xanthydryl.

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feeding and sexual behavior).[8] The actions of CRF are mediated through binding to CRF receptors, several of which have been characterized recently.[9−15] These receptors, like those for growth hormone-releasing factor, calcitonin and vasoactive intestinal peptide, are coupled via G-proteins and have seven putative transmembrane domains. The actions of CRF can also be modulated by a 37-kDa CRF binding protein (CRF-BP).[16] ACTH release from the pituitary is mediated by CRF1 receptors. Conditions characterized by too little or too much CRF might be alleviated by the administration of long-acting CRF agonists or antagonists, respectively. Structure−activity relationship studies presented here were designed to identify those residues that may modulate receptor activation, recognition, and binding. It is now well-documented that conformational constraints may modulate activities (turn an agonist into an antagonist or turn a ubiquitous hormone into a receptor-selective analogue).

While in early publications we proposed that CRF binding conformation(s) would be mostly α-helical,[17] we have subsequently hypothesized[1−3] that covalent constraints, such as side-chain-to-side-chain lactam rings, would stabilize such α-helical conformations. These studies led to the discovery of useful CRF antagonists (α-hel-CRF, Astressin) that are active both in vitro (rat pituitary cells in culture) and in the rat. To test the hypothesis that such lactam rings may also modulate activation of the receptor when introduced at the N-terminus of CRF, we studied the influence of the successive introduction from residues 4 to 14 of a cyclo-(i,i+3)[Lys^{i}−Glu$^{(i+3)}$] and a cyclo(i,i+3)[Glu^{i}−Lys$^{(i+3)}$] bridge on the in vitro potency of the agonist [Ac-Pro^{4},D-Phe^{12},Nle^{21,38}]hCRF$_{(4-41)}$ and related compounds. Early studies had demonstrated that deletion of residues 1−3 had no effect on biological activity or potency of CRF analogues. Also, while substitution of methionine residues at positions 21 and 38 of CRF antagonists by norleucine residues resulted in a significant increase in potency, it conferred chemical stability against oxidation as well.[18] Substitution of phenylalanine at position 12 by D-phenylalanine was also favorable in that it doubled the potency of ovine CRF.[18] Additionally, we reinvestigated the role of residues 6−8 for receptor activation in the presence of the favorable cyclo(Glu^{30}−Lys^{33}) modification. This reinvestigation was stimulated by several observations. We had shown that successive deletion of residues at the N-terminus of oCRF resulted in drastic loss of agonist potency with retention of full intrinsic activity (i.e., 1.0 and 0.9 for oCRF$_{(6-41)}$ and oCRF$_{(7-41)}$ relative to that of oCRF, while the relative potency of these analogues was 0.1 and 0.005, respectively).[18] Further deletion from the N-terminus led to loss of intrinsic activity (intrinsic activity of oCRF$_{(9-41)}$ was less than 10% that of oCRF). Similarly, but not quite identically, α-hel-CRF$_{(7-41)}$, α-hel-CRF$_{(8-41)}$, and α-hel-CRF$_{(9-41)}$ had 50%, 15%, and <10% intrinsic activities, respectively.[18] This suggested that substitutions preceding position 9 could influence intrinsic activity. This was confirmed with the more recent observation that the introduction of a cycle between the side chains of Glu^{30} and Lys^{33} led to little change in the potency of CRF agonists and a dramatic increase in the potency of CRF antagonists.[2] The role of residues 6−8, however, remained to be clarified.

Results and Discussion

All analogues shown in Table 1 were synthesized on a methylbenzhydrylamine resin using the Boc strategy with orthogonal protection of the side chains of lysine (Fmoc) and glutamic acid (OFm) residues to be cyclized.[1,19] Main-chain assembly was mediated in most cases by diisopropylcarbodiimide (DIC). The best results were obtained when the peptide chain was assembled in its entirety prior to cleavage of the Fmoc and OFm protecting groups and when TBTU or BOP mediated the lactam formation.[1] The peptides were cleaved and deprotected in HF and purified using RP-HPLC and three aqueous buffers (TEAP 2.25, TEAP 4.5−6.5, and 0.1% TFA).[1,20,21] The critical step in obtaining highly purified CRF analogues was the use of a TEAP buffer at a pH higher than 4.5. Under those conditions, impurities in amounts close to 30%, and difficult to detect otherwise, could be eliminated. Although very difficult to demonstrate, it is becoming apparent that those impurities (probably no single species is present in amounts greater than 1%) interfere with sensitive in vitro and in vivo assays. Peptides were characterized as shown in Table 1. Analogues were determined to be greater than 90% pure using RP-HPLC and CZE criteria. The measured masses obtained using liquid secondary ion mass spectrometry were in agreement with those calculated for the protonated molecule ions.

CRF analogues were tested for agonist activity in an in vitro assay measuring release of ACTH by rat anterior pituitary cells in culture.[4,22,23] Because the studies were carried out over a number of years, relative potencies with 95% confidence limits in parentheses are shown using either oCRF or hCRF as the assay standard with a potency equal to 1.0 (Table 1). The potencies of **1** and **20**, relative to that of hCRF, are about one-half and equal to that obtained when using oCRF as the standard, respectively. Since this difference is not statistically significant, and because it has been shown that oCRF and hCRF were essentially equipotent in this assay,[24] we will not distinguish between potencies found using oCRF or hCRF as standards. Additionally, six compounds (**1**, **20**, **21**, **24**, **26**, and **28**) were tested more than once to give consistent relative potencies, thus validating assay-to-assay reproducibility.

Even-numbered compounds **2**−**18** and odd-numbered compounds **3**−**19** belong to a cyclo(i,i+3)[Lys^{i}−Glu$^{(i+3)}$] and a cyclo(i,i+3)[Glu^{i}−Lys$^{(i+3)}$] scan, respectively, covering the N-terminus region of CRF. We showed in earlier studies that the cyclo(i,i+3)[Glu^{i}−Lys$^{(i+3)}$] substitution was more likely to yield potent analogues than the cyclo(i,i+3)[Lys^{i}−Glu$^{(i+3)}$] substitution. This observation was derived from the study of CRF antagonists[3] but did not apply to CRF agonists. The question remained whether inversion of the direction of the lactam bridge could, per se, modulate the ability of such molecules to activate the CRF receptor. Backbone amide bonds have been shown to mediate receptor activation since reduction to the backbone secondary amine yields antagonists in a number of systems such as cholecystokinin[25] and bombesin.[26] Results from the first scan {cyclo(i,i+3)[Lys^{i}−Glu$^{(i+3)}$]} yielded analogues

Table 1. Characterization of CRF Agonists by MS, HPLC, CZE, and Relative Potency

	5	10	15	20	25	30	35	40
Human/Rat CRF	S E E P P I S L D L T F H L L R E V L E M A R A E Q L A Q Q A H S N R K L M E I I NH_2							
human Urocortin	D N P S L S I D L T F H L L R T L L E L A R T Q S Q R E R A E Q N R I I F D S V-NH_2							
Sauvagine	pE G P P I S I D L S L E L L R K M I E I E K Q E K E K Q Q A A N N R L L L D T I-NH_2							
sucker Urotensin	N D D P P I S I D L T F H L L R N M I E M A R I E N E R E Q A G L N R K Y L D E V-NH_2							
α-helical-CRF	S Q E P P I S L D L T F H L L R E M L E M A K A E Q E A E Q A A L N R L L L E E A-NH_2							

ID#	Compound	MS (mono)[a] Calc.	MS (mono)[a] Obser.	Purity HPLC[b]	Purity CZE[c]	In vitro Potencies[d]
1	[Ac-Pro^4,DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4416.49	4416.5	98	>95	6.6 (4.5-9.9) (o)[e] 2.6 (1.4-4.8) (h) 2.5 (1.0-6.4) (h)
2	cyclo(4-7)[Ac-Lys^4,Glu^7,DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4471.53	4471.5	98	*97	0.05 (0.03-0.09) (o)
3	cyclo(4-7)[Ac-Glu^4,Lys^7,DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4471.53	4471.5	>98	*96	0.15 (0.08-0.30) (h)
4	cyclo(5-8)[Ac-Pro^4,Lys^5,Glu^8,DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4445.48	4445.5	95	93	0.001 (0.001-0.002) (o)
5	cyclo(5-8)[Ac-Pro^4,Glu^5,Lys^8,DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4445.48	4445.5	98	95	0.018 (0.008-0.046) (o)
6	cyclo(6-9)[Ac-Pro^4,Lys^6,Glu^9,DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4427.50	4427.4	95	92	0.6 (0.27-1.4) (o)
7	cyclo(6-9)[Ac-Pro^4,Glu^6,Lys^9,DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4427.50	4427.4	97	*98	0.25 (0.13-0.53) (h)
8	cyclo(7-10)[Ac-Pro^4,Lys^7,Glu^{10},DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4455.50	4455.5	95	96	0.003 (0.001-0.007) (o)
9	cyclo(7-10)[Ac-Pro^4,Glu^7,Lys^{10},DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4455.50	4455.5	98	97	0.004 (0.002-0.007) (o)
10	cyclo(8-11)[Ac-Pro^4,Lys^8,Glu^{11},DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4441.48	4441.6	95	95	0.0004 (0.00-0.001) (o)
11	cyclo(8-11)[Ac-Pro^4,Glu^8,Lys^{11},DPhe^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4441.48	4441.5	>98	*92	0.001 (0.000-0.002) (o)
12	cyclo(9-12)[Ac-Pro^4,Lys^9,Glu^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4393.52	4392.8	>98	96	0.002 (0.001-0.003) (o)
13	cyclo(9-12)[Ac-Pro^4,Glu^9,Lys^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4393.52	4393.6	97	96	0.011 (0.006-0.017) (o)
14	cyclo(9-12)[Ac-Pro^4,Lys^9,DGlu^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4393.52	4393.5	>98	>90	0.018 (0.008-0.036) (o)
15	cyclo(9-12)[Ac-Pro^4,Glu^9,DLys^{12},Nle^{21,38}]hCRF$_{(4-41)}$	4393.52	4393.2	92	90	1.2 (0.53-2.6) (h)
16	cyclo(10-13)[Ac-Pro^4,Lys^{10},DPhe^{12},Glu^{13},Nle^{21,38}]hCRF$_{(4-41)}$	4405.47	4405.5	>98	97	0.07 (0.03-0.16) (o)
17	cyclo(10-13)[Ac-Pro^4,Glu^{10},DPhe^{12},Lys^{13},Nle^{21,38}]hCRF$_{(4-41)}$	4405.47	4405.6	>98	*90	1.7 (1.0-3.1) (o)
18	cyclo(11-14)[Ac-Pro^4,Lys^{11},DPhe^{12},Glu^{14},Nle^{21,38}]hCRF$_{(4-41)}$	4441.48	4441.7	98	93	0.0004 (0.000-0.001) (o)
19	cyclo(11-14)[Ac-Pro^4,Glu^{11},DPhe^{12},Lys^{14},Nle^{21,38}]hCRF$_{(4-41)}$	4441.48	4441.5	98	>98	1.4 (0.85-2.36) (o)
20	cyclo(30-33)[Ac-Pro^4,DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(4-41)}$	4440.52	4440.4	>98	95	6.3 (3.2-12.9) (o) 4.3 (2.5-7.8) (h)
21	[Ac-Pro^4,DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(4-41)}$	4458.54	4458.6	96	95	4.5 (2.7-7.6) (h)[e] 4.0 (2.3-7.2) (h)
22	[Ac-Pro^4,DPhe^{12},Nle^{21},Lys(Ac)^{33},Nle^{38}]hCRF$_{(4-41)}$	4499.56	4499.6	96	92	3.10 (1.55-6.57) (h)
23	cyclo(30-33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(5-41)}$	4301.46	4301.5	97	97	2.6 (1.4-5.2) (h)

Table 1 (Continued)

24	cyclo(30-33)[Ac-Pro^5,DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(5-41)}$	4343.47	4343.5	98	*96	6.3 (3.5-11.8) (h)e 4.2 (2.5-7.2) (h)
25	cyclo(30-33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(6-41)}$	4204.41	4204.5	98	>98	4.0 (2.0-9.1) (h)
26	cyclo(30-33)[Ac-Ile^6,DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(6-41)}$	4246.42	4246.4	>98	>98	10.2 (4.7-24.3) (h) 4.3 (1.9-11.0) (h)
27	cyclo(30-33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(7-41)}$	4091.32	4091.4	98	95	1.35 (0.74-2.6) (h)
28	cyclo(30-33)[Ac-Ser^7,DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(7-41)}$	4133.34	4133.4	98	>98	8.1 (3.5-21.6) (h)e 3.0 (1.4-6.3) (h) 113% IA
29	cyclo(30-33)[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(8-41)}$	4004.29	4004.4	96	96	0.31 (0.19-0.50) (h) 65% IA
30	cyclo(30-33)[Ac-Leu^8,DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(8-41)}$	4046.30	4046.6	98	>98	1.20 (0.77-1.86) (h) 70% IA
31	[Ac-Leu^8,DPhe^{12},Nle^{21,38}]hCRF$_{(8-41)}$	4022.27	4022.3	>98	>98	0.01 (0.004-0.03)(h) 59% IA
32	cyclo(29-32)[DLeu^{11},Nle^{17},Glu^{29},Lys^{32}]Sau	4576.61	4576.7	95	>98	5.73(2.6-13.5) (o)
33	[DLeu^{11},Nle^{17},Glu^{29},Lys^{32}]Sau	4594.62	4594.6	97	97	0.53 (0.30-0.93) (h)e 0.54 (0.25-1.1)
34	cyclo(30-33)[DPhe^{12},Nle^{18,21},Glu^{30},Lys^{33}]sUtn	4829.53	4829.8	96	96	2.9 (1.3-6.6) (h)?
35	[DPhe^{12},Nle^{18,21},Glu^{30},Lys^{33}]sUtn	4847.52	4847.6	97	95	1.1 (0.6-2.3) (h)
36	hUrocortin	4694.51	4694.3	97	97	3.1 (1.4-6.7) (h)
37	rUrocortin	4705.52	4705.4	>98	>98	2.9 (1.5-6.9) (h)
38	cyclo(29-32) [Ac-Pro^3,DPhe^{11},Glu^{29},Lys^{32}]hUcn$_{(3-40)}$	4462.42	4462.5	95	95	3.8 (2.5-5.8) (h)
39	[Ac-Pro^3,DPhe^{11},Glu^{29},Lys^{32}]hUcn$_{(3-40)}$	4480.43	4480.3	95	95	1.3 (0.83-1.9) (h)
40	cyclo(30-33)[Ac-Pro^4,DPhe^{12},Nle^{18,21},Glu^{30},Lys^{33}]α-hel-CRF$_{(4-41)}$	4336.36	4336.4	95	94	7.5 (4.6-12.2) (h)
41	[Ac-Pro^4,DPhe^{12},Nle^{18,21}]α-hel-CRF$_{(4-41)}$	4354.37	4354.4	>98	96	1.3 (0.77-2.2) (h)
42	cyclo(30-33) [Ac-Leu^8,DPhe^{12},Nle^{18,21},Glu^{30},Lys^{33}]α-hel-CRF$_{(8-41)}$	3942.10	3942.1	>98	>98	0.073 (0.032-0.17)(h) 93% IA
43	[Ac-Leu^8,DPhe^{12},Nle^{18,21}]α-hel-CRF$_{(8-41)}$	3944.15	3944.6	>98	>98	0.004 (0.002-0.008)(h)

[a] The observed *m/z* of the monoisotope compared with the calculated $[M + H]^+$ monoisotopic mass. [b] Percent purity determined by HPLC using buffer system: A, TEAP (pH 2.5); B, 60% CH_3CN/40% A; with a gradient slope of 1% B/min, at a flow rate of 0.2 mL/min on a Vydac C_{18} column (0.21 × 15 cm, 5-μm particle size, 300-Å pore size). Detection at 214 nm. [c] CZE was done using a Beckman P/ACE System 2050 controlled by an IBM Personal System/2 model 50Z and using a ChromJet integrator. Field strength of 15 kV at 30 °C, mobile phase of 100 mM sodium phosphate (85:15, H_2O/CH_3CN), pH 2.50, on a Supelco P175 capillary (363-μm o.d. × 75-μm i.d. × 50-cm length). Detection at 214 nm. *Some CZE had to be done in the presence of 30% acetonitrile, for hydrophobic analogues. [d] Potencies are relative to ovine CRF$^{(o)}$ and/or rat/human CRF$^{(h)}$ in the in vitro rat pituitary cell culture assay, with 95% confidence limits in parentheses. IA as defined in the Experimental Section. [e] These particular compounds were tested two or three times.

with potencies ranging from very low (below 1%: **4**, **8**, **10**, **12**, and **18**), to low (around 5%: **2**, **14**, and **16**), and to one-half that of the standard (**6**). All analogues displayed high intrinsic activities suggesting that none could act as competitive antagonists. Results from the second scan {cyclo(i,i+3)[Glu^i−Lys$^{(i+3)}$]} also yielded analogues with potencies ranging from very low (below 1%: **9** and **11**) to low (around 2%: **5** and **13**), to approximately 5 times less than that of the standard (**3** and **7**), and to being as potent as the standard (**15**, **17**, and **19**). All of these analogues displayed high intrinsic activities, suggesting that none could act as

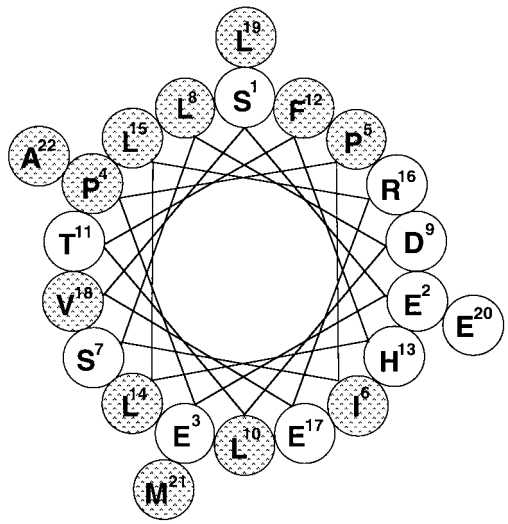

Figure 1. Edmunson wheel of the N-terminus 22 residues of CRF.

competitive antagonists. These data therefore preclude the possibility that, in these series (**2**−**19**), the lactam bridge interferes with receptor activation. In most cases, such constraints are deleterious to potency and therefore to binding affinity, with three exceptions (**15**, **17**, and **19**). It is particularly telling that in these three compounds, the direction of the amide bond parallels that of the backbone and involves residues that follow the critical residues (5−8), identified earlier as those likely to trigger receptor activation. From these data we conclude that the introduction of structural constraints by both lactam rings described here did not qualitatively affect the ability of these analogues to activate the pituitary CRF receptors. The fact that these substitutions had quantitative effects suggests that other factors, such as the ability of CRF1 to recognize particular residues, may play a preponderant role as indicated earlier by systematic studies such as alanine,[27] D-amino acid,[18] and single-point slight alteration scans.[28]

The possibility that maintenance of a preferred conformation is necessary for receptor activation and that this preferred conformation is modulated by a number of substitutions cannot be excluded. If CRF assumes an α-helical structure through the N-terminus, albeit likely disrupted at Pro,[4,5] all of the residues in positions 1−12 are observed to be parts of larger substructures differentiated by hydrophobicity (see Figure 1). As shown by modeling studies, starting at the N-terminus, residues Ser^{1}, $Glu^{2,3}$, Ser^{7}, and Thr^{11} form a contiguous polar patch. This patch is in registration with a previously described[1] polar patch involving Glu^{25}, $Gln^{26,29,30}$, and Ser^{33}. On the N-terminus axial face, opposing the patch just described, is a relatively highly charged continuous patch involving Asp^{9}, Glu^{12}, His^{13}, Arg^{16}, Glu^{20}, and Arg^{23}. Separating these two elongated stretches of hydrophilic residues emanating from the N-terminus are two stretches of enhanced hydrophobicity involving residues Ile^{6}, $Leu^{10,14}$, Val^{18}, Ala^{22} and Leu^{8}, Phe^{12}, Leu^{15}, Leu^{19}, Ala^{22}. Both of these elongated patches terminate at Ala^{22}. Consequently, if an idealized α-helix is a good model of CRF's receptor interactions, then the individual N-terminal residues are responsible for the hydrophobic/hydrophilic interactions with specific regions of the receptor. To test this hypothesis, we deleted, one at a time (with and without acetylation), N-terminal amino acids after residue 4 in cyclo(30−33)[Ac−Pro^{4},DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]-$hCRF_{(4-41)}$ (**20**). This analogue (**20**) was reported earlier to have a potency of 6.3 (3.2−12.9), whereas the corresponding unbridged analogue (**21**) had a potency of 4.5 (2.7−7.6), and the corresponding linear and blocked analogue at positions 30 (Gln) and 33 (Lys(Ac)) also had a potency of 3.1 (1.6−6.6), all relative to that of hCRF = (1.0).[2] The fact that these three analogues are essentially equipotent suggests that they most likely interact with pituitary CRF receptors in a similar fashion with equal affinity. The next six analogues with deletion of residues 1−4 (**23**, **24**), 1−5 (**25**, **26**), and 1−6 (**27**, **28**) (without and with acetylation of the N-terminus, respectively) were surprisingly potent, with the exception of **27** which is 4−5 times less potent than the others. The fact that these analogues are significantly more potent than the 1−5 deletion analogues reported earlier ($oCRF_{(6-31)}$ is 10% as potent as oCRF)[22] reinforces an earlier hypothesis that the lactam cyclization (cyclo(30−33)) maintains a structural constraint in these shorter analogues that would otherwise be normally induced by the N-terminal amino acids. It is also interesting to note some loss of potency after deletion of residues 1−6 (**27**) and that potency is restored by acetylation (**28**). It is well-known that blocking the N-terminus may result in helix stabilization.[29,30] Also consistent is the observation that deletion of one additional residue (Ser^{7}, **29**) leads to a 5-fold decrease in potency as compared to **27** and some loss of intrinsic activity (65% IA). Interestingly, acetylation of the N-terminus of **30** (70% IA) restores most of the potency and not the intrinsic activity, suggesting, in a different way, that a stronger macrodipole (destabilized in **29** by a positive charge at the N-terminus) will stabilize the helical bioactive conformation and therefore increase affinity. Considerably more significant is the loss of potency of **31** (100-fold) but not of intrinsic activity (59% IA) compared to that of **30** upon loss of the stabilizing cycle at positions 30−33. This loss is of the same order of magnitude as that observed between cyclic and linear antagonists which are shortened by an additional residue as compared to **29**−**31**.

At this point we wanted to confirm that the observations made with analogues of hCRF could be paralleled with analogues of other members of the CRF family. We have shown that except for α-hel-CRF and urocortins (**36**, **37**), which are 3−8 times more potent than human CRF,[22,31] all are known to be equipotent with CRF in the assay described here.[32,33] In fact, in the case of sauvagine, going from the favored cyclic analogue **32** (lactam ring mediated by Glu^{29}−Lys^{32}) to the corresponding linear analogue **33** results in a 10-fold loss of potency which is greater (2-, 2-, 2-, and 5-fold, respectively) than what had been observed in the case of hCRF (**20**, **21**), sucker urotensin (**34**, **35**), human urocortin (**38**, **39**), or α-hel-$CRF_{(4-41)}$ (**40**−**41**) upon loss of the same structural constraint.

Finally, deletion of residues 1−7 in α-helical CRF, yielding **42** and **43** with potencies of 7% and 0.4% (20-fold difference), shows that the introduction of residues promoting α-helicity somewhat compensates for the loss

of potency seen when comparing the effect of the same deletion in hCRF analogues **30** and **31** (100-fold difference). This is in agreement with our earlier conclusion that cyclization at positions 30−33 (or equivalent, i.e., 29−32 in sauvagine and urocortin) maintained the analogues in a conformation favorable for binding and activation of the receptor.

Of great interest now is the observation that deletion of one additional residue leads to extremely potent antagonists (J. Rivier et al., in preparation, and ref 22) suggesting a unique role of the leucine/isoleucine residues at position 8 for activation of the CRF receptor. Either of these two residues is highly conserved throughout the members of the CRF family.

Experimental Section

Synthesis of CRF Analogues. All analogues shown in Table 1 were synthesized either manually or on a Beckman 990 peptide synthesizer using the solid-phase approach, a 4-methylbenzhydrylamine resin,[1] and the Boc strategy with orthogonal protection (Fmoc and OFm) of the side chains of residues to be cyclized.[19] Amino acid derivatives Boc-Ala, Boc-Arg(Tos), Boc-Asn(Xan), Boc-Asp(cHex), Boc-Gln(Xan), Boc-Glu(cHex), Boc-His(Tos), Boc-Ile, Boc-Met, Boc-Leu, Boc-Phe, Boc-Pro, Boc-Ser(Bzl), Boc-Thr(Bzl), Boc-Tyr(2-Br-Cbz), and Boc-Val were obtained from Bachem Inc. (Torrance, CA), Chem-Impex Inc. (Wood Dale, IL), and Calbiochem (San Diego, CA). Boc-Glu(Ofm) and Boc-Lys(Fmoc) were synthesized as described earlier.[34] All solvents were reagent grade or better. TFA, 50−60% in DCM (1% *m*-cresol), was used to remove the Boc group. Main-chain assembly was mediated by DIC. Three-fold excess protected amino acid was used based on the original substitution of the 4-methylbenzhydrylamine resin. When the synthesis was carried out on a synthesizer, coupling time was 90−120 min followed by recoupling after residue 32 (with the exception of glycine and alanine residues which were not recoupled). Automatic acetylation (excess acetic anhydride in DCM for 15 min) was carried out after addition of each amino acid. When analogues were synthesized manually, recouplings were carried out only when necessary and acetylations only when recoupling could not yield negative ninhydrin tests.[35] Deprotection of the Fmoc group was achieved using a fresh solution of 20% piperidine/DMF or NMP (2 × 10 min) followed by sequential washes with DMF, MeOH, 10% TEA/DCM, and DCM. Lactam formation was mediated using BOP, TBTU, or HBTU in DMF or NMP. Best results were obtained when the peptide chain was assembled in its entirety prior to cleavage of the Fmoc and Ofm protecting groups and cyclization as shown earlier.[1] The peptides were cleaved and deprotected in HF in the presence of anisole (5−10%, v/v) and purified using RP-HPLC and three solvent systems (TEAP at pH 2.25 and 6.5 and 0.1% TFA, successively).[20,21]

Characterization of CRF Analogues. Peptides were characterized as shown in Table 1. Analogues were greater than 90% pure using independent HPLC and CZE criteria. Conditions are shown in the legend.

RP-HPLC: In addition to determining the purity of the peptides in an acidic system (see Table 1 legend), most of the analogues were also analyzed using 0.05% TEAP at pH 6.8 and a Vydac C_8 column (0.21 × 15 cm) at a flow rate of 0.2 mL/min with slightly varying gradient slopes. Retention times varied from 14 to 33 min with no correlation between apparent hydrophobicity and relative potency. Percent purity was in the range of that found with CZE or with HPLC under acidic conditions.

Capillary Zone Electrophoresis (CZE): CZE was carried out using a Beckman P/ACE System 2000 controlled by an IBM Personal System/2 model 50Z and using a ChromJet integrator. Electrophoresis was performed in 0.1 M sodium phosphate (pH 2.5) except for α-hel-$CRF_{(9-41)}$ which was measured in 0.1 M sodium borate (pH 8.5).

Mass Spectroscopy: LSIMS mass spectra were measured with 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; for further details, see ref 1. Calculated values for protonated molecular ions were in agreement with those observed using liquid secondary ion mass spectrometry.

In Vitro Pituitary Cell Culture Assay: Rat anterior pituitary glands from male Sprague−Dawley rats were dissociated by collagenase treatment and plated (0.16×10^6 cells/well in 48-well plates) in medium containing 2% fetal bovine serum (FBS).[23] Three days after plating, the cells were washed three times with fresh medium containing 0.1% bovine serum albumin (BSA) and incubated for 1 h. Following the 1-h preincubation, the cells were washed once more and the test peptides were applied. At the end of a 3-h incubation period, the media were collected and the level of ACTH was determined by radioimmunoassay (Diagnostic Products Corp.). Intrinsic activity is the ratio of the levels of ACTH released at maximal doses by the analogues and CRF, respectively, times 100. When not mentioned IA = 100%.

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