Corticotropin Releasing Factor (CRF) Agonists with Reduced Amide Bonds and Ser^7 Substitutions

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Strategies to generate competitive antagonists of bioactive peptides include several possible structural modifications such as the introduction of D-residues and of reduced amide bonds, the substitution of amino acid side chains, dimerization of fragments, and deletion of part of the sequence, among others. Whereas we have identified the two most likely residues responsible for receptor activation in corticotropin releasing factor (CRF) (Ser^7 and Leu^8)[1] and generated potent antagonists by deleting residues 1−8,[2,3] the question remained as to whether we could generate CRF antagonists with enhanced affinity after reduction of amide bonds at the N-terminus of CRF or through subtle alteration of those residues' side chains. Reduced amide bond replacements (ψ[CH_2NH]) between residues 6−9 in oCRF$_{(5-41)}$ (**11**, **12**, **15**) analogues consistently yielded potencies of <1% that of oCRF. Except for the $^{10}\psi^{11}$ and $^{12}\psi^{13}$ analogues 19 and 20, reduced amide bond replacements were generally well-tolerated in the longer hCRF$_{(4-41)}$ analogues, with the $^{7}\psi^{8}$-, $^{8}\psi^{9}$-, and $^{9}\psi^{10}$-modified peptides (**13**, **14**, **18**) yielding potencies that were 2−4 times that of hCRF. Although somewhat promising as agonists, they were, however, 3−7 times less potent than the parent [D-Pro^{4}Nle^{21,38}]-hCRF$_{(4-41)}$ (**2**). Since O-alkylation of Tyr^3 in vasopressin yields an antagonist, and since Ser^7 is one of the eight fully conserved residues in the CRF family (inclusive of sauvagine, urocortins, and urotensins) and likely to be critical for receptor binding, we synthesized cyclo(30−33)[Ser(OMe)^7,D-Phe^{12},-Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$ (**22**), which was found to exhibit full efficacy and 40% of the potency of cyclo(30−33)[D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$ (**5**). Other substitutions at position 7 included aminoglycine (**23**, **24**) and alkylated and/or acylated [α or α′-methyl (**25**−**28**), α′-formyl (**29**, **30**), α′-formyl, α′-methyl (**31**), α′-acetyl (**32**), α′-acetyl, α′-methyl (**33**)], D- or L-aminoglycines. All analogues were active although less potent than the parent compound **2**, and all elicited maximal ACTH response as compared to hCRF. The most potent analogue in this series (**33**) had the bulkiest side chain, Agl(Me, Ac), and was 60% and 80% as potent as the Ser^7 analogue **5** and the Ala^7 analogue **6**, respectively. In conclusion, we found that neither reduction of the individual amide linkages between residues 6−11 and 12−13 nor introduction of a carbamide moiety in lieu of the side chain of Ser^7 led to CRF antagonists.

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 (review[7]). CRF was also subsequently found in extra-hypothalamic 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 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 hormone, calcitonin, and vasoactive intestinal peptide, among others, are coupled via G-proteins and have seven putative transmembrane domains. The actions of CRF are also 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.

In an earlier paper, we identified residues Ser^7 and Leu^8 as the residues that might modulate receptor activation, recognition, and binding.[1] Indeed, deletion of residues 1−8 in CRF and urotensins and of residues 1−7 in sauvagine and urocortins yielded potent competitive antagonists.[2,17] We have also shown that binding affinity can be increased by the introduction of D-Phe^{12} and a Glu^{30}-Lys^{33} side chain to side chain bridge.[18] In our quest for even more potent CRF

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antagonists, we investigated other approaches to peptide competitive antagonism. Strategies to generate competitive antagonists of bioactive peptides include several broad classes of chemical modifications such as the introduction of D-residues[19,20] and of reduced amide bonds,[21,22] substitution of amino acid side chains,[23] dimerization of fragments,[24] and deletions of part of the sequence.[2,25] We have found no evidence for antagonists or partial agonists in either a D-amino acid scan[26] or an alanine scan[27] of oCRF. The question remained, however, as to whether introduction of a reduced amide bond at any position between residues 6−12 would result in lowered efficacy with retention of binding affinity. We had identified Ser^7 of CRF and urotensins and Ser^6 of sauvagine and urocortins,[3] as being one of the eight conserved residues (Asp^9, Leu^{10}, Leu^{15}, Arg^{16}, Ala^{31}, Asn^{34}, and Arg^{35}, using CRF's numbering scheme) in CRF as being critical for receptor activation. To determine the role of the side chain of Ser^7, we investigated the effects of methylation of the hydroxyl function or substitution of residue 7 by acylated and/or alkylated aminoglycines.

Results and Discussion

All peptides shown in Table 1 were assembled either manually or on a Beckman 990 synthesizer using the *tert*-butoxycarbonyl (Boc)-amino acid strategy on a *p*-methylbenzhydrylamine (MBHA)[28,29] resin with orthogonal protection of the side chains of lysine (Fmoc) and glutamic acid (OFm) residues to be cyclized.[30,31] Briefly, couplings were mediated by diisopropylcarbodiimide (DIC), benzotriazolyloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), or *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) in dichloromethane (DCM), dimethylformamide (DMF), or *N*-methylpyrrolidinone (NMP) for 2 h. The Nα-Boc groups were removed with trifluoroacetic acid (TFA), and the Fmoc/OFm side chain protecting groups were removed with 20% piperidine in dimethylformamide (DMF) or *N*-methylpyrrolidinone (NMP). Lactam cyclization was performed after Fmoc deprotection of the side chains of the bridgehead residues using BOP, HBTU, or TBTU for coupling. Reduced amide bonds were introduced using the method of Hocart et al.[32] by in situ reductive alkylation of the peptide N-terminus when treated with the α-amino aldehyde and excess $NaBH_3CN$. Boc-protected amino aldehydes were synthesized from the parent amino acids in two steps using the method of Fehrentz and Castro.[33] First, the *N*-methoxy-*N*-methylamide was formed from the amino acid and *O*,*N*-dimethylhydroxylamine. The amides were purified by flash chromatography and characterized by optical rotation, infrared spectroscopy, and melting point, when possible. In the next step, the amides were reduced to aldehydes with $LiAlH_4$. All aldehydes except for Boc-Ala-CHO (solid) were oils. Boc-Ala-CHO, Boc-Ile-CHO, Boc-Leu-CHO, Boc-Phe-CHO, and Boc-Ser(OBzl)-CHO were characterized for purity by thin-layer chromatography, and their IR and optical rotations were compared to literature data. Oils and solids were used rapidly and without purification in the reductive alkylation step before decomposition could occur. During these studies we experienced synthetic difficulties with Thr which, once introduced in the desired sequence, yielded irreproducible results as shown by mass spectrometry. Additionally, we could not repeat in hCRF (introduction of $Asp^9\psi[CH_2NH]Leu^{10}$) the results of Hocart et al.,[32] describing the introduction of a reduced $Asp^3\psi[CH_2NH]$-Ala^4 bond in human growth hormone releasing hormone.

Peptides **23**−**33** that incorporated derivatized α-aminoglycine moieties were synthesized according to the procedure of Jiang et al.[34,35] After lactam cyclization, racemic Boc-Agl(Fmoc), Boc,Me-Agl(Fmoc), or Boc-Agl(Me, Fmoc)[36] was coupled to the peptide−resin with BOP in 50% NMP/DCM. The appropriate nitrogen was deblocked and derivatized by either acetylation or formylation as desired. Final deprotection to produce a free nitrogen in **23**−**28** was performed prior to peptide cleavage from the resin and side chain deprotection using HF. Since derivatization of Agl moieties produced two diastereomeric peptides, we routinely used the nomenclature (L or D) and (D or L) to denote the hydrophilic and hydrophobic diastereomers, respectively, as they eluted on RP-HPLC under acidic conditions. These pseudonyms in no way reflect the true absolute configurations of these centers, since the X-ray structure of a direct antecedent has not yet been determined for Agl-containing peptides. Additionally, we have shown that the HPLC retention times of single-point D-amino acid-substituted peptides can be shorter or longer than that of the corresponding L-amino acid-substituted peptide.[26]

All peptides were cleaved and deprotected with hydrogen fluoride. Purification was achieved to >95% purity, in most cases, via RP-HPLC on a C_{18} column with a gradient of TEAP/CH_3CN buffers at pH 2.25 or 6.5, followed by 0.1% TFA/CH_3CN buffers.[37] The purified peptides were characterized by quantitative HPLC, capillary zone electrophoresis (CZE), and mass spectral analysis (see Table 1). Analogue **31** was unresolvable by preparative HPLC and was determined to be a 3:1 ratio of the L- and D-isomers by analytical HPLC using the TEAP 2.3 buffer. It should be noted that the bis-acetylated Agl in **32** is achiral, thus yielding a single compound. On the other hand, **33** appeared as a single entity by HPLC and CZE, which indicates that it may consist of one or two diastereomers. The measured masses obtained using liquid secondary ion mass spectrometry were generally in agreement with those calculated for the protonated molecule ions. Difficulties were encountered with the analysis of several of the Agl-containing analogues. In particular, the LSI-, ESI-, and MALD-MS of compounds **27**, **28**, and **31**, measured in the positive ionization mode, contained intense fragment ions corresponding with loss of either 72 or 60 Da. In each case, these compounds contain disubstituted nitrogen atoms (the N-terminal nitrogen of **27** and **28** are methylated and acetylated, while the side chain aminoglycine of **31** is methylated and formylated). Curiously, compound **33** which also contains a disubstituted nitrogen atom was found to be stable under both the LSI-MS and MALD-MS conditions used for the analysis of the above compounds. In the case of compound **31**, we were able to observe an intense intact molecule ion

Table 1. Characterization of CRF Agonists by HPLC, CZE, MS, and Relative Potency in Primary Culture of Rat Pituitary Cells

	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	Q	A	H	S	N	R	K	L	M	E	I	I	NH_2

no.	peptide structures	% purity HPLC[a]	% purity CZE[b]	MH^+ (mono)[c] calcd	MH^+ (mono)[c] obsd	in vitro potency[d]	ref
1.	hCRF	>97[III]	>97	4755.51	4755.5	1.00 standard	38
2.	[D-Pro^4,Nle^{21,38}]hCRF$_{(4-41)}$	>97[III]	97	4374.48	4374.4	11 ± 2[‡,††]	
3.	[Nle^{21,38}]Ac-hCRF$_{(5-41)}$	>97[III]		4319.44	4319.4	0.36 (0.1−7.0)[††]	
4.	[D-Phe^{12},Nle^{21,38}]Ac-hCRF$_{(7-41)}$	>97[I]	92	4109.30	4109.5	1.5 (0.68−3.4)[†]	
5.	cyclo(30−33)[D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	>97[I]	>97	4133.34	4133.4	5.5[‡‡,†]	1
6.	cyclo(30−33)[Ala^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	96[II]	>97	4117.34	4117.3	4.3 (2.1−9.5)[†]	
7.	[D-Phe^{12},Nle^{21,38}]Ac-hCRF$_{(8-41)}$	>97[I]	>97	4022.27	4022.3	0.01 (0.004−0.028)[†] MR 59%[e]	1
8.	cyclo(30−33)[D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(8-41)}$	>97[I]	>97	4046.30	4046.6	1.2 (0.77−1.9)[†] MR 70%[e]	1
9.	[D-Pro^4,Ile$^6\psi$[CH$_2$NH]Ser^7,Nle^{21,38}]hCRF$_{(4-41)}$	97[I]	95	4360.50	4360.5	0.31 (0.12−0.71)[††]	
10.	[D-Pro^4,Ile$^6\psi$[CH$_2$NH]Ser^7,Tyr^{11},Nle^{21,38}]hCRF$_{(4-41)}$	>97[III]	97	4422.51	4422.5	0.021 (0.007−0.049)[††]	
11.	[Ile$^6\psi$[CH$_2$NH]Ser^7,Nle^{21}]Ac-oCRF$_{(5-41)}$	>97[III]	>97	4237.38	4237.4	0.0016 (0.000−0.004)[††]	
12.	[Ser$^7\psi$[CH$_2$NH]Leu^8,Nle^{21}]Ac-oCRF$_{(5-41)}$	>97[III]	>97	4237.38	4237.3	0.003 (0.001−0.009)[††]	
13.	cyclo(30−33)[D-Pro^4,Ala$^7\psi$[CH$_2$NH]Leu^8,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(4-41)}$	91[I]	>97	4368.54	4368.6	3.2 (1.7−5.8)[†]	
14.	[D-Pro^4,Leu$^8\psi$[CH$_2$NH]Asp^9,Nle^{21,38}]hCRF$_{(4-41)}$	97[III]	94	4360.50	4360.4	2.1 (0.92−5.0)[††]	
15.	[Leu$^8\psi$[CH$_2$NH]Asp^9,Nle^{21}]Ac-oCRF$_{(5-41)}$	>97[III]	97	4237.38	4237.4	0.007 (0.002−0.02)[††]	
16.	cyclo(30−33)[Leu$^8\psi$[CH$_2$NH]Asp^9,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(8-41)}$	>97[I]	>97	4032.32	4032.3	0.4 (0.2−0.8)[†] MR 60%[e]	
17.	cyclo(30−33)[D-Pro^4,Ala^9,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]-hCRF$_{(4-41)}$	97[I]	95	4354.52	4354.6	3.7 (1.2−15)[†]	
18.	cyclo(30−33)[D-Pro^4,Ala$^9\psi$[CH$_2$NH]Leu^{10},D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]-hCRF$_{(4-41)}$	94[I]	96	4340.54	4340.7	7.0 (4.1−11.5)[†]	
19.	[D-Pro^4,Leu$^{10}\psi$[CH$_2$NH]Thr^{11},Nle^{21,38}]hCRF$_{(4-41)}$	97[III]	>97	4360.50	4360.4	0.10 (0.05−0.19)[††]	
20.	[D-Pro^4,Phe$^{12}\psi$[CH$_2$NH]His^{13},Nle^{21,38}]hCRF$_{(4-41)}$	>97[III]	>97	4360.50	4360.4	0.005 (0.002−0.010)[††]	
21.	cyclo(30−33)[D-Ser7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	>97[I]	>97	4133.34	4133.4	3.1 (1.8−5.2)[†]	
22.	cyclo(30−33)[Ser(OMe)^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	>97[I]	>97	4147.34	4147.4	2.2 (1.1−4.4)[†]	
23.	cyclo(30−33)[(L or D)-Agl^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	97[I]	93	4118.34	4118.3	0.16 (0.096−0.25)[†]	
24.	cyclo(30−33)[(D or L)-Agl^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	97[I]	>97	4118.34	4118.2	0.1 (0.06−0.15)[†]	
25.	cyclo(30−33)[(L or D)-Agl(Me)^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	>97[I]	>97	4132.35	4132.6	0.28 (0.13−0.57)[†]	
26.	cyclo(30−33)[(D or L)-Agl(Me)^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	97[I]	97	4132.35	4132.4	1.9 (0.96−3.8)[†]	
27.	cyclo(30−33)[(L or D)-MeAgl^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	82[I]	80	4132.35	4060.3	0.33 (0.21−0.51)[†]	
28.	cyclo(30−33)[(D or L)-MeAgl^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	70[I]	86	4132.35	4060.3	0.21 (0.12−0.34)[†]	
29.	cyclo(30−33)[(L or D)-Agl(For)^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	90[I]	92	4146.33	4146.4	0.85 (0.41−1.7)[†]	
30.	cyclo(30−33)[(D or L)-Agl(For)^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	95[I]	>97	4146.33	4146.4	0.54 (0.36−0.81)[†]	
31.	cyclo(30−33)[D/L-Agl(Me,For)^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	3/1[I] ratio	95	1386.3	1386.7*	0.58 (0.24−1.3)[†]	
32.	cyclo(30−33)[Agl(Ac)^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	>97[I]	>97	4160.35	4160.5	0.21 (0.10−0.44)[†]	
33.	cyclo(30−33)[D/L-Agl(Me,Ac)^7,D-Phe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF$_{(7-41)}$	97[I]	>97	4174.36	4174.3	3.4 (1.7−6.9)[†]	

[a] Percent purity was determined by HPLC using one of three buffer systems selected for giving the best resolution: (I) A = TEAP (pH 2.5); (II) A = TEAP (pH 6.8); (III) A = 0.1% TFA. [b] Conditions for capillary zone electrophoresis (CZE) are described in the Experimental Section. [c] Calculated and observed *m/z* values of the $[M + H]^+$ monoisotopes are reported. All observed *m/z* were measured using LSI-MS unless otherwise noted by an asterisk, in which case the observed unresolved $[M - 3H]^{3-}$ species was measured using ESI-MS and corresponds closely with the calculated molecule mass (M = 4161.90). [d] Agonist potencies are relative to that of hCRF[†] or oCRF[††] = 1 in the in vitro rat pituitary cell culture assay with 95% confidence limits in parentheses. [‡]Potency of **2** is the average of three assays ± SEM [relative potencies were 14 (8.4−25), 11 (2.7−56), and 8.7 (4.7−17)]. [‡‡]Potency of **5** is the average of two assays [8.1 (3.4−22) and 3.0 (1.4−6.3)]. [e] The percent maximal ACTH release (MR) of each of the partial agonists **7**, **8**, and **16** is calculated by determining the level of secretion caused by the highest dose of agonist minus basal secretion and dividing that number by the level of secretion of 1 nM hCRF minus basal secretion and multiplying the result by 100.

under ESI-MS negative ionization mode conditions, thereby demonstrating that the species observed under positive ionization conditions was a fragment ion. In the case of **27** and **28**, the observed fragment ions correspond with facile loss of $(CH_3CO)(CH_3)N^{\bullet}$ (72 Da).

CRF analogues were tested for agonist activity in an in vitro assay measuring release of ACTH by collagenase-dispersed rat anterior pituitary cells in culture.[2,4] Due to the fact that studies were carried out over a number of years, the potencies are relative to that of either oCRF or hCRF (potencies equal to 1.0), which were shown earlier to be equipotent in this system with an $EC_{50} = 0.043 \pm 0.012$ nM.[38,39] Confidence limits (95%) are shown in parentheses.

We have shown in earlier publications that deletion of the N-terminal residues (1−3)[4] up to (1−7)[3] yielded agonists with somewhat reduced potencies as the size of the peptide decreased. For example, whereas oCRF, Ac-oCRF, and $oCRF_{(4-41)}$ were equipotent, $oCRF_{(6-41)}$ and $oCRF_{(7-41)}$ had 11% and 0.5% of oCRF's potency, respectively.[2] Our current data demonstrate that the loss of residues 1−4 proves to be deleterious to potency (**3** is one-third as potent as hCRF), despite acetylation and methionine replacement by Nle. However, increasing the peptide length to (4−41) by the addition of D-Pro[4] increases the potency 33-fold (compare the potencies of **2** and **3**). This suggests an important role for Pro[4]. Such observations led us to delete residues 1−3 in most of our SAR investigations and to acetylate Pro[4] or substitute it with D-Pro[4]. The additional substitution of Phe[12] by D-Phe[12] is clearly favorable in **4**, as it restores some potency despite further shortening of the sequence to (7−41).

Recently, our studies showed that introduction of a lactam bridge between Glu[30] and Lys[33] dramatically improved potencies of CRF antagonists but also improved potencies of shortened agonists to a greater degree than it did of full length agonists. A conclusion was that the lactam bridge linking the side chains of residues 30−33 favored a conformation that was otherwise induced by the presence of residues 4−8.[18] Compounds **7** and **8** illustrate the effects of different substitutions and further chain shortening of the CRF sequence on potency. Comparing the potencies of **4** and **7** clearly points to the importance of residue 7 since **7** is 150 times less potent than **4**. Although we[26,27] and others[40] concluded from earlier studies that Ser at position 7 was critical, the substitution of Ser[7] of **5** by Ala[7] in **6** results in no loss of potency. In summary, we have identified four favorable modifications in $CRF_{(7-41)}$ and $CRF_{(8-41)}$ analogues: acetylation of the N-terminus, substitution of Phe[12] by D-Phe[12], substitution of both methionines by Nle, and the introduction of a 30−33 cycle. Incorporation of these in **8** (as compared to only three modifications in **7**) is a way to retain full potency, although with lowered efficacy. Similar observations had led us to conclude that residues 7 and 8 are critical for binding to the active form of the CRF receptor.[3]

As mentioned earlier, the question remained as to whether introduction of a reduced amide bond at any position between residues 6−12 would result in lowered efficacy and retention of binding affinity in a manner both distinct from and less detrimental to binding affinity than deletion of residues 1−8[2] and 1−11.[41] Individual SAR analysis of each bond gives surprising results. First, reduction of the amide bond between residue Ile[6] and Ser[7] results in a loss of potency for **9** (>30-fold loss vs **2**) and **11** (>200-fold loss vs **3**). Although **9** and **11** retain 30 and 0.2% of the potency of oCRF respectively, the significance of these potencies becomes more pronounced when compared to those of the peptide templates as noted. Thus, such a large potency loss occurs in each case despite the precedent of the potent 4−41 (**2**) template and is dramatically magnified in the less potent 5−41 (**3**) template. Additionally, we substituted Thr[11] by Tyr to determine its effect on the potency of **9**. We observed from earlier studies that Thr[11] is sensitive to substitutions,[27,40] yet hoped that maintenance of a hydroxyl group (aromatic versus aliphatic) at that position would have only a limited effect on potency. The fact that **10** is ca. 15-fold less potent than **9** corroborates these studies on the sensitivity of Thr[11] to substitutions.

Reduction of the amide bond between residues Ser[7] or Ala[7] and Leu[8] (**12** and **13**, respectively) yields peptides with potencies that are 0.3% that of oCRF (**12**) and 3 times that of hCRF (**13**). When compared to its closest template **3**, the $oCRF_{(5-41)}$ analogue **12** is ca. 100 times less potent than **3**. Since oCRF and hCRF are equipotent in this assay, we assume that their Nle-substituted fragments would also be equipotent; if this was the case, these data suggest that reduction of the amide bond between residues 7 and 8 is very deleterious. However, cyclic $hCRF_{(4-41)}$ analogue **13** is equipotent to its template **6**, which opposes this argument. Precedence indicates that deletion of residues 1−6 at the N-terminus of cyclic(30−33)CRF analogues is not detrimental since cyclo(30−33)[D-Phe[12],Nle[21],Glu[30],-Lys[33],Nle[38]]Ac-$hCRF_{(4-41)}$ is equipotent to cyclo(30−33)-[D-Phe[12],Nle[21],Glu[30],Lys[33],Nle[38]]Ac-$hCRF_{(7-41)}$.[1] Therefore, we can also hypothesize that reduction of the amide bond between residues 7 and 8 is not significant. This contrasts with the observation that linear **12** is 100 times less potent than **3** which indicated (pending some assumptions) that the amide bond was indeed important for potency. As both analogues with reduced amide bonds between residues 7 and 8 exhibited full efficacy and since we were interested in developing a novel approach to CRF antagonists, we did not pursue further isosteric bond modifications at Ser[7] and Leu[8].

Reduction of the amide bond between residues Leu[8] and Asp[9] (**14**−**16**) gives parallel results to those seen with the reduction of the amide bond between residues 7 and 8. Following a similar trend, the $^{8}\psi^{9}$-$hCRF_{(4-41)}$ **14** remains equipotent to oCRF whereas the $^{8}\psi^{9}$-$oCRF_{(5-41)}$ **15** retains less than 1% the potency of oCRF. When comparing these analogues to their closest templates, we also observe a 5-fold loss of potency for the $hCRF_{(4-41)}$ analogue **14** (as compared to **2**) and a significant loss of potency (ca. 50-fold as compared to **3**) for the $oCRF_{(5-41)}$ ovine analogue **15**. Interestingly, the shorter, cyclic $hCRF_{(8-41)}$ analogue **16** retains 40% of the potency of hCRF. Yet, when comparing the potency of **16** to that of **8**, we find that the 3-fold decrease must be assigned to the introduction of the $^{8}\psi^{9}$ reduced amide bond. Of interest is the fact that **7**, **8**, and **16** show lowered efficacies than the CRF standards

(59−70%), a property that we can assign to the deletion of residues 1−7.

We were unable to introduce the reduced amide bond between Asp[9] and Leu[10] using the conditions described here; therefore, we synthesized **17** with Ala at position 9 and its reduced amide homologue **18**. Whereas [Ala[9]]-oCRF[27] and [Glu[9]]oCRF[40] were 25 and ca. 5 times less potent than oCRF, respectively, the same Ala[9] substitution in the cyclic **17** was well-tolerated; additionally, Ala[9]ψ[CH$_2$NH]Leu[10] had no deleterious effect on potency (in fact, **18** is twice as potent as **17**). This was in contrast with the 100- and 1000-fold loss of potency compared to that of **2** resulting from reduction of the amide bond between residues 10−11 (**19**) and 12−13 (**20**).

In summary, analogues with reduced amide bonds (except for **16**) all released ACTH maximally, although their potencies varied significantly. We conclude that the reduced amide bond modification shown to yield antagonists in a number of bioactive peptides[21,42] is not a strategy that we could apply to CRF analogues by targeting the N-terminal residues known to be responsible for binding to the activated form of the receptor. We do not know the effect of such a modification at residues of the C-terminus, a region of the molecule responsible for binding to the G-protein coupled and G-protein uncoupled forms of the receptor. We have shown with agonist and antagonist radioligands that the latter detect more mature receptors than do the agonists even though their K_ds are the same. This has been explained by the hypothesis that agonists only bind to the fraction of receptors that are G-protein coupled. Antagonists, on the other hand, bind to the G-protein coupled and uncoupled receptors.[43]

Another approach to the design of antagonists is to modify an amino acid chirality or side chain in such a way that it prevents binding to the activated form of the receptor but not to that of the inactivated form. For example, this is the result of the introduction of a D-Cys[3] in somatostatin analogues[20] or of O-alkylation of [Tyr[3]] in vasopressin[44] which yields antagonists. Since Ser[7] is one of the eight fully conserved residues in the CRF family (inclusive of sauvagine, urocortins, and urotensins) and also sensitive to substitutions, we hypothesized that subtle modifications of that residue might indeed yield important information with regard to the mechanism of binding of CRF to its receptor(s). We synthesized cyclo(30−33)[D-Ser[7],D-Phe[12],Nle[21],Glu[30],-Lys[33],Nle[38]]Ac-hCRF$_{(7-41)}$ (**21**) and cyclo(30−33)[Ser-(OMe)[7],D-Phe[12],Nle[21],Glu[30],Lys[33],Nle[38]]Ac-hCRF$_{(7-41)}$ (**22**) that elicited a maximal ACTH response and ca. 50% the potency of cyclo(30−33)[D-Phe[12],Nle[21],Glu[30],Lys[33],Nle[38]]-Ac-hCRF$_{(7-41)}$ (**5**) or 2−3 times the potency of hCRF. This was an unexpected result in view of earlier data showing that [D-Ser[7]]oCRF, [Ala[7]]oCRF, and [Thr[7]]-oCRF had less than 15% the potency of oCRF.[26,27,40]

We have shown recently that substitution of most residues in acyline (Ac-D-Nal-D-Cpa-D-Pal-Ser-Aph(Ac)-D-Aph(Ac)-Leu-ILys-Pro-D-Ala-NH$_2$), a potent GnRH antagonist, by their corresponding acylated-aminoglycines[35] yielded analogues with potencies that were not statistically different from that of acyline itself.[34] We have also shown that substitution of His[2] in TRH by Agl(2-imidazole-carboxyl) and other closely related acylated aminoglycines yields inactive analogues.[45] Continuing with our focus on 7-position analogues, we substituted Ser[7] with Agl (**23**, **24**) or alkylated/acylated [α or α'-methyl (**25**−**28**), α'-formyl (**29**, **30**), α'-formyl, α'-methyl (**31**), α'-acetyl (32), α'-acetyl, α'-methyl (**33**)] D- or L-aminoglycines.[7] The purpose of introducing an acylated and/or an alkylated nitrogen linked to the α-carbon of glycine was to constrain rotational freedom about the side chain that might influence receptor binding. We found that all analogues were potent, some (**26**, **29**, and **33**) were as potent and others (**23**−**25**, **27**, **28**, **30**−**32**) were less potent (minimum of 10% potency) than hCRF. None of these substituted analogues showed lowered efficacy (indicative of partial agonism), which suggests that the side chain functionality of Ser[7] does not drastically influence receptor binding.

In conclusion, we have hypothesized that residues 1−12 of CRF may be important for binding to the G-protein coupled form of the CRF receptor and that subtle substitution in that part of the molecule may yield a unique family of antagonists. In an earlier paper,[1] we had narrowed the critical residues down to Ser[7] and, to a lesser extent, Leu[8]. Here we reassessed the importance of the N-terminus of CRF in receptor binding using two different strategies that independently address the role of the peptide backbone (using a reduced amide bond scan) and that of Ser[7] (using aminoglycine derivatives). Some of the substitutions resulted in loss of potency, while others had little effect; yet only the CRF$_{(8-41)}$ analogues had lowered efficacy (ca. 60%).

Since so little is known with respect to peptide antagonist design, it is still possible that a reduced amide scan of the entire peptide backbone or the introduction of an acylated aminoglycine at a critical position would yield desired antagonists. From a structural point of view, the most successful approaches consist of introducing structural constraints such as side chain to side chain bridges or methylation of selected α-carbons.[1,17] Thus the increased backbone flexibility imparted by a reduced amide bond opposes structural restrictions; yet this modification has been shown to provide resistance to enzymatic degradation.[46] In addition, we also showed here that substitution of Ser[7] by acylated aminoglycines and methylated homologues known to limit side chain flexibility has limited effect on the potency of cyclo(30−33)CRF agonists and no effect on efficacy.

Experimental Section

Instruments and Methods. The HF cleavage line was designed in-house and allowed for HF distillation under high vacuum. Preparative HPLC were run on a Waters Prep 500 with model 500A preparative gradient generator, model 450 variable wavelength UV detector, PrepPAK 1000, and Fisher Recordall 5000 strip chart recorder. The 5 cm × 30 cm cartridge was packed in the laboratory with reversed-phase 300 Å Vydac C$_{18}$ silica (15−20 μm particle size, THE SEP/A/RA/TIONS GROUP). Analytical HPLC screening was performed on a Vydac C$_{18}$ column (0.46 × 25 cm, 5 μm particle size, 300 Å pore size) connected to a Rheodyne injector, two Waters M-45 pumps, a Waters automated gradient controller, Kratos SF 7697 UV detector, Shimadzu Chromatopac E1A integrator, and Houston Instruments D-5000 strip chart recorder. Quality control HPLC was performed on one of two systems: (1) Peptides that were analyzed in TFA, as indicated

in Table 1, were screened on a Waters Associates HPLC system comprised of two 6000A pumps, a WISP sample injector, a Kratos Spectroflow model 773 UV detector, and a Waters Associates data module integrator/recorder. (2) Peptides that were analyzed in TEAP were screened on a Hewlett-Packard Series II 1090 liquid chromatograph, Controller model 362, and a Think Jet printer. CZE analysis was performed on a Beckman P/ACE System 2050 controlled by an IBM Personal System/2 model 50Z connected to a ChromJet integrator.

Starting Materials. The *p*-Methylbenzhydrylamine−resin (MBHA−resin) with a capacity of 0.35−0.75 mequiv/g was obtained from a polystyrene cross-linked with 1% divinylbenzene (Biobeads SX-1, 200−400 mesh, Bio-Rad Laboratories, Richmond, CA) as previously published.[29] All Nα-Boc-protected amino acids with side chain protection were purchased from Bachem Inc. (Torrance, CA), Calbiochem (San Diego, CA), or Chem-Impex Intl. (Wood Dale, IL). The side chain protecting groups were as follows: Arg(Tos), Asn(Xan), Asp(β-OcHex), Gln(Xan), Glu(γ-OcHex), Glu(γ-OFm), His(Tos), Lys(ϵ-2ClZ), Lys(ϵ-Fmoc), Ser(OBzl), Ser(OMe), Thr(OBzl), Tyr(2BrZ). Reagents and solvents were analytical reagent grade.

Peptide Synthesis. Peptides were made by the solid-phase approach[28,47] either manually or on a Beckman 990 peptide synthesizer. Couplings on 1−2 g of resin per peptide were mediated for 2 h by diisopropylcarbodiimide (DIC) in DCM, and DMF or NMP was used for Boc-protected Arg(Tos), Asn(Xan), Gln(Xan), His(Tos), Ile, and Leu. Synthetic progress was monitored by the qualitative ninhydrin test.[48] Difficult couplings were mediated with BOP, HBTU, or TBTU in DMF or NMP and adjusted to pH 9 with diisopropylethylamine. Unprotected Boc-Asn and Boc-Gln were coupled in the presence of 1.5 equiv of HOBt. A 3-fold excess of amino acid based on the original substitution of the resin was used in most cases. Coupling steps were followed by acetylation [10% $(CH_3CO)_2O$ in DCM for 10−15 min and 1 drop of pyridine] as necessary. Boc removal was achieved with trifluoroacetic acid (50% in DCM, 1−2% ethanedithiol or *m*-cresol) for 20 min. An isopropyl alcohol (1% ethanedithiol or *m*-cresol) wash followed TFA treatment, and then successive washes with triethylamine (TEA) solution (10% in DCM), methanol, TEA solution, methanol, and DCM completed the neutralization sequence. Lactam cyclization was performed after complete peptide assembly whereupon the Fmoc side chain protecting groups were removed with 20% piperidine in DMF or NMP in two successive 10 min treatments. The method of Felix et al.[49] was followed using BOP, HBTU, or TBTU as the coupling agent. The peptides were cleaved and deprotected in HF in the presence of 10% anisole and 2−5% dimethyl sulfide (for Met-containing peptides) for 1.5 h at 0 °C. After HF distillation, the crude peptides were precipitated with diethyl ether, filtered, and dissolved in 10% aqueous acetic acid or 25% aqueous CH_3CN. The products were then shell-frozen and lyophilized.

Synthesis of Reduced-Amide-Containing Peptides. Couplings proceeded as detailed above until the amino acid to be reduced was reached, whereupon the Boc-α-amino aldehyde was coupled following the procedure of Hocart et al.[50] on the same 0.25 mmol scale. Boc-Ala-CHO, Boc-Ile-CHO, Boc-Leu-CHO, Boc-Phe-CHO, and Boc-Ser(OBzl)-CHO were synthesized following the procedure of Fehrentz and Castro[33] on a scale of 2−5 mmol. Following Boc-group removal at the N-terminus and TFA neutralization wash, the peptide resin was slurried with 1% AcOH in NMP, and 3 equiv of Boc-α-amino aldehyde were added. The resin was mixed for 45 min, and then $NaBH_3CN$ (10 equiv) was added at once. The reaction proceeded overnight (18 h) at room temp, and then the resin was drained and washed well with 1% AcOH/NMP, MeOH, 10% TEA/DCM, MeOH, and DCM. Qualitative ninhydrin testing determined reaction completion. The standard protocol for peptide solid-phase synthesis was then resumed to complete the peptide assembly. Yields for reduced amide peptides after purification were 10−50 mg.

Synthesis of Agl-Containing Peptides. Racemic Boc-Agl(Fmoc), Boc,Me-Agl(Fmoc), and Boc-Agl(Me, Fmoc) were kindly provided by G. Jiang as previously published.[36] Since the Agl derivatives were the last amino acids to be coupled to the resin, the lactam formation between residues **30** and **33** was completed prior to the final Agl(R) (R = H, Me) coupling and derivatization. The protected Agl(R) was coupled to 0.25−0.5 mmol of peptide−resin using standard protocol with BOP in 50% NMP/DCM, adjusted to pH 9 with 3 equiv of diisopropylethylamine for 2−3 h. In some cases recouplings were necessary until a satisfactory ninhydrin test resulted. The appropriate nitrogen was deblocked and derivatized by either acetylation or formylation ($HCO_2H + Ac_2O$, premixed at 0 °C then added to resin in NMP for 2−3 h) as desired.[34] Yields of final product were 3−30 mg.

Purification.[37] The crude, lyophilized peptides (1−3 g) were dissolved in a minimum amount (300 mL) of 0.25 N TEAP, pH 2.25, and CH_3CN and loaded onto the preparative HPLC. The peptides eluted with a flow rate of 100 mL/min using a linear gradient of 1% B per 3 min increase from the baseline %B. (Eluent A = 0.25 N TEAP, pH 2.25; eluent B = 60% CH_3CN, 40% A.) Generally, purifications in TEAP, pH 2.25, followed by TEAP, pH 6.5, were necessary to achieve the desired purity level. As a final step, the TEAP salt of the peptide was exchanged for the TFA salt using a gradient of 1% B/min where A = 0.1% TFA.

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

1. RP-HPLC. Peptide purity was determined by analytical HPLC in either 0.1% TFA or TEAP buffer systems as indicated in Table 1. The 0.1% TFA system was defined by a 1% B/min gradient slope from equilibrium A/B where A = 5% CH_3CN/0.1% TFA and B = 80% CH_3CN/0.1% TFA on a Vydac C_{18} column (0.46 × 25 cm, 5 μm particle size, 300 Å pore size), flow rate of 1.5−2.0 mL/min, detection at 214 nm. The TEAP system was defined by a 1% B/min gradient slope from equilibrium A/B where A = 15 mM TEAP, pH 2.3 (pH 6.8 for 6), and *B* = 60% CH_3CN/40% A on a Vydac C_{18} column at 40 °C (0.21 × 15 cm, 5 μm particle size, 300 Å pore size), flow rate of 0.2 mL/min, detection at 214 nm.

2. Capillary Zone Electrophoresis (CZE). CZE analysis employed a field strength of 15 kV at 30 °C with a buffer of 100 mM sodium phosphate/25% CH_3CN at pH 2.5 on either a Beckman eCAP or a Supelco P175 fused silica capillary (363 mm o.d. × 75 mm i.d. × 50 cm length).

3. Mass Spectroscopy. LSI-MS measurements were carried out 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 between 25 and 30 kV were employed. The samples were added directly to a glycerol and 3-nitrobenzyl alcohol (1:1) matrix. The mass of each analogue was measured, and the observed monoisotopic $(M + H)^+$ values were consistent with the calculated $(M + H)^+$ values with the exceptions outlined in the text. MALD-MS measurements were carried out on a Bruker Reflex (Bruker Daltonics, Billerica, MA) reflectron time-of-flight mass spectrometer utilizing a nitrogen UV laser and 100 MHz digitizer. The instrument was operated with an accelerating voltage of +31 kV and reflector potential of +30 kV. The mass spectrum represented the accumulation of approximately 20 laser shots. ESI-MS measurements were carried out using an Esquire ion trap mass spectrometer (Bruker Daltonics, Billerica, MA). The HPLC purified sample, redissolved in 0.1% aqueous TFA and CH_3CN, was diluted in methanol 1% AcOH, infused at 500 nL/min, and analyzed. The mass accuracy was typically better than ±1000 ppm for the time-of-flight instrument, ±200 ppm for the ion trap instrument, and ±100−20 ppm for the double-focusing mass spectrometer depending on the resolving power settings of the magnetic sector instrument employed.

4. In Vitro Pituitary Cell Culture Assay. Rat anterior pituitary glands from male Sprague−Dawley rats were dissociated by collagenase treatment and plated (0.16 × 106 cells/well in 48-well plates) in medium containing 2% fetal bovine serum.[3] Three days after plating, the cells were washed 3 times

with fresh medium containing 0.1% bovine serum albumin and incubated for 1 h. Following the 1 h preincubation, the cells were washed once more and the test peptides were applied. Typically the analogues are dissolved in 100 mM AcOH−0.1%BSA at a concentration of 200 mM and then further diluted in 1 mM AcOH−0.1%BSA. This vehicle alone does not elicit an ACTH response. The standard agonist assay always includes a dose−response curve for CRF which ranges from 0.002 to 5 nM in increments of 5. All analogues are tested within the same range, possibly higher or lower depending on their expected potencies. At the end of a 3 h incubation period, the media were collected and the level of ACTH was determined by radioimmunoassay (Diagnostic Products Corporation). Each point of each curve is done in triplicate, and all values are used in the assessment of potency. Intra- and interassay variabilities in the ACTH-RIA were less than 10%. Maximal response is the ratio of the levels of ACTH released at maximal doses by the analogues and CRF, respectively, times 100. When not mentioned, MR = 100%. To determine potencies, the dose−response curve of the test analogue is compared to that of CRF. Potencies of agonists were calculated against reference standards hCRF or oCRF using the BIOPROG program,[51] which generates one curve for each analogue that was compared to that of the corresponding standard. The curves must be parallel in order for the potency to be valid. Assays are repeated in the rare cases when nonparallelism is found.

Abbreviations

IUPAC rules are used for nomenclature of peptides including one letter codes for amino acids. Also, Ac = acetyl; ACTH = adrenocorticotropin hormone; Agl = aminoglycine; Aph = 4-aminophenylalanine; 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; Cpa = 4-chlorophenylalanine; CRF = corticotropin releasing factor (o = ovine, h = human); CZE = capillary zone electrophoresis; DCM = dichloromethane; DIC = diisopropylcarbodiimide; DMF = dimethylformamide; ESI-MS = electrospray mass spectroscopy; Fmoc = 9-fluorenylmethoxycarbonyl; HBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HF = hydrogen fluoride; Ilys = N^{ε}-isopropylysine; LSI-MS = liquid secondary ion mass spectrometry; MALD-MS = matrix-assisted laser desorption mass spectroscopy; MBHA = methylbenzhydrylamine resin; MR = maximal ACTH response = efficacy; Nal = 2-naphthylalanine; NMP = *N*-methylpyrrolidinone; OFm = *O*-fluorenylmethyl; Pal = 3-pyridylalanine; SEM = standard error of the mean; TBTU = *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TEA = triethylamine; TEAP = triethylammonium phosphate; TFA = trifluoroacetic acid; Xan = xanthyl; ψ[CH$_2$NH] or $^{x}\psi^{z}$ = reduced amide bond between *x* and *z* residues.

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