NMR Structural Characterization of a Minimal Peptide Antagonist Bound to the Extracellular Domain of the Corticotropin-releasing Factor, Receptor*†

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Natural peptide agonists of corticotrophin-releasing factor (CRF) receptors bind to the receptor by a two-site mechanism as follows: the carboxyl end of the ligand binds the N-terminal extracellular domain (ECD) of the receptor and the amino portion of the ligand binds the extracellular face of the seven transmembrane region. Recently, peptide antagonists homologous to the 12 C-terminal residues of CRF have been derived, which bind the CRF₁ receptor through an interaction with the ECD. Here we characterized the binding of a minimal 12-residue peptide antagonist while bound to the isolated ECD of the CRF₁ receptor. We have expressed and purified soluble and properly folded ECD independent from the seven-transmembrane region as a thioredoxin fusion protein in Escherichia coli. A model of the peptide antagonist, cyclic corticotrophin-releasing factor residues 30–41 (cCRF₃₀–₄₁), was calculated while bound to the recombinant ECD using transferred nuclear Overhauser effect spectroscopy. Although the peptide is unstructured in solution, it adopts an α-helical conformation when bound to the ECD. Residues of cCRF₃₀–₄₁ comprising the binding interface with the ECD were mapped using saturation transfer difference NMR. Two hydrophobic residues (Met₃₈ and Ile₄₁) as well as two amide groups (Asn₃₄ and the C-terminal amide) on one face of the helix defined the binding epitope of the antagonist. This epitope may be used as a starting point for development of non-peptide antagonists targeting the ECD of this receptor.

Corticotropin-releasing factor₁ (CRF₁)² receptor plays a crucial modulatory role in the central stress response, making it an attractive target for the development of therapeutics for a variety of stress-related disorders, including anxiety and depression (1–5). The CRF₁ receptor is a class B G-protein-coupled receptor (GPCR) and is activated endogenously in mammals by the peptide agonists CRF (6) and the urocortins (7–9). Binding of the native ligands to the receptor promotes a conformational change that leads to G-protein activation inside the cell. Signaling is mediated primarily through the second messenger cAMP via Goᵢ; however, coupling of the receptor to Goᵢ and Goᵢq₁₁ has been observed (10, 11). The receptor is expressed both in the periphery, where pituitary activation underlies the major role of CRF activation of the hypothalamic pituitary adrenal axis during stress, and in brain, where extrahypothalamic CRF activates central receptors to mediate mood and affect (12).

A two-site binding and activation model has been proposed for agonist binding to class B GPCRs (13). Recent pharmacological data suggest that activation of the CRF₁ receptor is initiated by the C terminus (≈30 amino acids) of the peptide ligand interacting with relatively moderate affinity to the extracellular N-terminal domain (ECD) of the CRF₁ receptor (14). This primary interaction has the effect of increasing the local concentration of the ligand and positions its N terminus (≈10 amino acids) in the optimal orientation to interact with the juxtamembrane region of the receptor, thus inducing the conformation change in the receptor required for activation and signaling. The model has been built through a series of binding and mutagenesis studies of both the CRF₁ receptor and its ligands (14–19). One such study showed that a deletion mutant of the CRF₁ receptor lacking the entire ECD was still capable of signaling but only in the presence of high (micromolar) concentrations of CRF (14). This observation supported the two-site binding model hypothesis suggesting that the role of the ECD appears to be crucial in “trapping” the agonist ligand thus achieving physiologically relevant concentrations to activate the receptor (14, 16, 17, 20).

Peptide antagonists derived from native ligands have played a major role in the current level of understanding of class B GPCRs. For the CRF₁ receptor, it was shown that truncation of the N-terminal 8 residues (CRF₉–₄₁) results in a loss of agonism but not the ability to bind to the receptor, thus creating an antagonist (21). The remaining C-terminal sequence of CRF was predicted and demonstrated to be predominantly α-helical in the presence of helix-stabilizing additives (22). This finding was applied in the design of the potent peptide antagonist astressin. The increased potency of astressin versus CRF₉–₄₁...
was achieved through selective residue substitutions and by introducing a covalent constraint between residues 30 and 33, which is presumed to stabilize the α-helical conformation (23). Although deriving most of its binding affinity from an interaction with the ECD of the CRF₁ receptor, it was recently shown that astressin can induce receptor internalization (24). In addition, a 10-fold decrease in the affinity of astressin binding was observed when the juxtamembrane region of the receptor was removed (14). Together, these facts imply that astressin interacts both with the ECD at high affinity, as well as with the juxtamembrane domain, albeit with lower affinity, of the receptor. This property distinguishes astressin from truncated peptide antagonists developed by Yamada and co-workers (25, 26), which have equivalent potencies to astressin but are significantly smaller (12 amino acids) and are pure competitive antagonists with no effect on receptor trafficking. Because these minimal peptide antagonists interact only with the ECD and because they have superior solubility characteristics versus astressin, CRF, or the urocortins, we selected one such peptide, cyclic corticotropin-releasing factor residues 30–41 (cCRF₃₀–₄₁), to study the molecular determinants of ligand binding to the ECD of the CRF₁ receptor. cCRF₃₀–₄₁ corresponds to the 12 C-terminal residues of human CRF with the exception of residues 30 and 33, which are cyclized by a lactam bridge (Fig. 1A). The binding affinity of cCRF₃₀–₄₁ was most appropriate for NMR study; however, the most potent version of this 12-residue peptide family (YAM19) is equipotent to astressin and is shown for comparison in Fig. 1B.

In these studies, binding of cCRF₃₀–₄₁ to the isolated ECD of human CRF₁ receptors was characterized by using a combination of NMR spectroscopy techniques (27–29). A model of cCRF₃₀–₄₁ was constructed while bound to this recombinant ECD using transferred nuclear Overhauser effects (trNOE), in which a sufficient off-rate of the peptide from the ECD allowed information about interproton distances to be derived from the bound state of the peptide (30, 31). In addition, the primary points of interaction with the ECD (the binding epitope) were characterized using saturation-transfer difference NMR (STD NMR) (32, 33). These findings suggest that the peptide is unstructured in solution and adopts an α-helical conformation upon binding to the ECD, burying a largely hydrophobic face that includes two amide functionalities.

**EXPERIMENTAL PROCEDURES**

**Synthetic Peptides**—Human CRF, astressin, biotin-astressin, YAM19, and cyclic CRF₃₀–₄₁ were synthesized by solid-phase methodology on a Beckman Coulter 990 peptide synthesizer using t-Boc-protected amino acids and then was HPLC-purified. Peptide quality was assessed by analytical HPLC and electrospray mass spectrometry.

**Biotinylated Peptides**—Using the above methodology, the N-terminal dPhe of astressin was replaced with dTyr-Gly(γ-biotin)-Lys to create biotinylated astressin (bAs). The affinity of bAs for full-length CRF₁ receptors was within 2-fold of unmodified astressin. Similarly, C-terminally deamidated astressin (bAS-OH) was produced which had ~2000-fold lower binding affinity than bAs and was used to generate our ligand affinity resin. Biotinylated CRF was purchased from Bachem AG (catalog number H-5756).

**Isotopically Labeled Peptides**—The cCRF₃₀–₄₁ peptide was isotope-labeled at specific amino acids with ¹⁵N and ¹³C using two separate schemes. The first scheme specifically labeled the residues Ala31, Asn34, Leu37, Met38, and Ile41. The second scheme uniformly labeled the residues Arg35, Leu37, Met38, and Ile41. Labeled reagents for the synthesis included the following: sodium acetate-d₅ (99.9%), acetic acid-d₆ (99.9%), and D₂O were purchased from Isotec, Inc. (St. Louis, MO). The l-[³-¹³C]alanine-N-t-Boc, l-[U-¹³C,¹⁵N]asparginine-N-Fmoc, N-β-trityl, l-[U-¹³C,¹⁵N]leucine-N-Fmoc, l-[methyl-¹³C]methionine, l-[¹,₂-¹³C]glutamic acid-N-t-Boc, γ-benzyl ester, and l-[U-¹³C,¹⁵N]isoleucine-N-Fmoc were purchased from Cambridge Isotope Laboratories (Andover, MA). The peptide used for the second labeling scheme was synthesized by Bachem AG (Bubendorf, Switzerland).

**Expression and Purification of trx-ECD**—Amino acids Ser¹–Ala₉⁶, comprising the entire N-terminal extracellular domain (ECD) (excluding the 23-amino acid signal peptide) of human CRF₁ receptor, were cloned into the pET-32a vector and expressed in Origami™ pLysS Escherichia coli as a C-terminal

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**FIGURE 1.** A, chemical structure of the cCRF₃₀–₄₁ peptide used in these studies. B, amino acid sequences of natural and derived peptide antagonists of the CRF₁ receptor. Affinities of these peptides for trx-ECD using the SPA assay were measured to be 3 μM and 17, 545, and 5.9 nM for CRF, astressin, cCRF₃₀–₄₁, and YAM19, respectively (14).
NMR Studies of CRF₄ Peptide Antagonist Binding

fusion protein with thioredoxin (trx). The trx-ECD fusion protein was expressed as inclusion bodies, which were solubilized with 6 M urea and purified by nickel-chelate chromatography (Invitrogen). Monomeric trx-ECD was then isolated by gel filtration chromatography using a HiPrep 26/60 gel filtration column (Amersham Biosciences). Properly folded trx-ECD was isolated by running the monomeric pool over a peptide ligand affinity column (bAS-OH immobilized on streptavidin-Sepharose). Finally, trx-ECD was purified by reverse-phase HPLC using a C4 column to remove salts and buffers, lyophilized, and stored at −20 °C until needed. Details of the expression and purification are provided in the Supplemental Material.

Blot Overlay Binding Assay—To analyze the results of ligand affinity chromatography, fractions containing trx-ECD were run on SDS-PAGE, transferred to Protran nitrocellulose (0.2 μm; Schleicher & Schuell), and visualized with Ponceau S protein stain to compare protein levels in each lane. After destaining, the blots were blocked with 5% milk in TTBS for 1 h and then incubated overnight at 4 °C with either 1 μM bAs or 5 μM biotinylated CRF. To reveal the extent of binding, the washed blots were incubated for 1 h at room temperature with horseradish peroxidase-conjugated streptavidin. The blots were then washed and developed with WestPico ECL substrate (Pierce) and exposed to film.

Scintillation Proximity Binding Assay (SPA)—Radioligand binding experiments were performed in SPA format using streptavidin-coupled polystyrene imaging beads (Amersham Biosciences). The trx-ECD fusion protein was biotinylated using the EZ-Link Sulfo-NHS-LC biotinylation kit (Pierce) and resulted in an average of two biotin labels per protein. Reactions were performed in singlet and consisted of 25 μl of unlabeled peptide at the indicated concentrations, 25 μl of radiolabeled [¹²⁵I]astressin (∼300 pm, 2,200 Ci/mmol; custom iodination from PerkinElmer Life Sciences), and 50 μl of trx-ECD/SPA beads added sequentially in assay buffer (phosphate-buffered saline, pH 7.4, 2 mM EGTA, 10 mM MgCl₂) to low binding 96-well plates (Corning Glass, Palo Alto, CA). The reaction was shaken and allowed to settle at room temperature for 90 min prior to reading with a CCD imaging system (Viewlux, PerkinElmer Life Sciences).

A single-site binding model fit best for all displacement binding experiments. \( K_a \) values were calculated using the Cheng-Prussoff equation with a sigmoidal dose-response (variable slope) fit using Prism 4.0 software (GraphPad Software, San Diego, CA) using \( K_a \) values of 10 nM for [¹²⁵I]astressin as determined from saturation binding experiments. Hill slopes for all curves routinely ranged from −0.9 to −1.2.

NMR Experiments—All NMR samples were prepared with a peptide concentration of 2 mM in 50 mM sodium acetate-\( d_4 \), pH 4.5, 90% H₂O, 10% D₂O + 0.01% NaN₃. Samples used for the trNOE experiment were 100 μM trx-ECD (20:1), and two-dimensional STD NMR experiments were performed with a 40 μM concentration of trx-ECD (50:1), and one-dimensional STD NMR for epitope mapping was done with 20 μM trx-ECD (100:1).

Spectra were obtained on a Bruker Avance 500-MHz spectrometer using a 4-mm SEI flow cell probe equipped with z axis gradients. Water suppression was achieved using the double-pulsed field gradient spin-echo scheme with 2-μs gaussian-shaped inversion pulses for selective inversion of the water resonance and a gradient ratio of 3:1 (34). Experiments were performed at 37 °C because the off-rate of the peptide at this temperature was more favorable for the trNOE experiments.

Sequential ¹H assignments for the peptide were obtained using standard methods (35). Spin systems were identified using a combination of two-dimensional ¹H-¹H total correlation spectroscopy and ¹H-¹H ROESY experiments. For this peptide, the intermediate correlation time (\( \tau \sim 1.12 \) ) necessitated the use of rotating-frame Overhauser effects (ROE) to characterize the free peptide. Additional information was obtained from two selectively ¹³C-labeled peptide samples. The free peptide was also characterized using the phase-sensitive two-dimensional ¹H-¹³C HSQC experiment (36).

Transferred NOE intensities were found to be linear up to 150 ms, so this data set was used for modeling of the bound structure. Mapping of the ECD-contacting residues was performed using one-dimensional saturation transfer difference (STD) NMR and employed 40 gaussian-shaped pulses of 50 ms each (for a total saturation time of 2 s) at alternating on- and off-resonance frequencies of −1 and 30 ppm, respectively (32). Mapping of the binding epitope was further characterized using this sample under the same conditions as the one-dimensional experiment using a two-dimensional STD HSQC experiment. Additional trNOEs were obtained from the ¹³C-labeled peptide using a two-dimensional ¹H-¹³C trNOESY-HSQC experiment. The two-dimensional total correlation spectroscopy-HSQC, ROESY-HSQC, trNOE-HSQC, and STD HSQC spectra are available in the Supplemental Material.

Data were processed using XWINNMR 3.5 running on a LINUX work station and analyzed using SPARKY (37). Peaks in the two-dimensional spectra were selected, and their volumes were quantitated from the 150-ms trNOE spectrum using the standard gaussian line-fitting algorithm. One-dimensional NMR spectra were processed and analyzed using the NMR Manager program from ACD/Labs (Ontario, Canada).

Modeling of Bound Peptide—Peak volumes obtained from trNOE spectra were used to create structural restraints that were introduced into a stochastic energy minimization protocol as implemented in MOE on a desktop PC (38). For the calculations described, the force field used was the CHARMM27 all-atom potential (39). To accurately simulate the environment of the peptide, an implicit solvation energy was calculated using the Born solvation model (40, 41). Target distance restraints were incorporated into the protocol based on the proton NOE intensities from the 150-ms trNOE experiments. NOE peaks were grouped as weak, medium, and strong, representing upper distance restraints of 5–6, 3–5, and <3 Å, respectively. Restraints for diastereotopic methylene protons were allowed to switch between protons; however, the configurations of the side chains were not found to vary with the protocol that was used because of the low number of long range distance information.

For these calculations, the protocol was initiated with 50 replicates of an extended template with the bond angles and distances from the standard CHARMM27 topology file. These conformations were the starting point for the series of stochastic searches in Cartesian space using the standard method dis-
tributed in MOE. The experimental restraints were added (along with the Born solvation term) to the potential using harmonic functions centered at the appropriate inter-hydrogen distance ranges. Diminishing torsion constraints were used to initially guide certain regions of the molecule to increased sampling of the α-helical region. These restraints were not found to be absolutely necessary but increased the efficiency of the search. Experimental and torsion restraints were relaxed in a series of 10 steps. Convergence of each run was determined by satisfaction of a standard geometric tolerance, residual energy based on the CHARMM27 potential, and the experimental restraints.

RESULTS
Preparation and Characterization of Functional trx-ECD—The N-terminal ECD of the CRF₁ receptor was fused to the C terminus of trx with an intervening His tag and thrombin cleavage sites and was expressed in E. coli. The strain of E. coli used to express the trx-ECD fusion protein has no functional thioredoxin reductase or glutathione reductase, and the resulting oxidative environment allows for the formation of disulfide bonds within the cytoplasm of the bacteria.

The expressed trx-ECD was present as inclusion bodies that were solubilized with urea and purified by nickel-chelate chromatography to remove contaminant proteins (Fig. 2A). When analyzed by SDS-PAGE, purified trx-ECD is a monomer in the presence of reducing agent, but in the absence of reducing agent, it migrates as both a monomer and a series of oligomers (Fig. 2A). Purified trx-ECD did not react in assays designed to detect free thiols (data not shown). These observations led us to conclude that disulfide bonding was complete and that both intra- and intermolecular disulfide bonds were present. Because properly folded ECD should contain three intramolecular disulfide bonds and no intermolecular disulfide bonds, we used gel filtration to separate out the oligomers and isolate the monomeric pool of trx-ECD (Fig. 2B). There are 15 possible combinations of intramolecular disulfide bonds for the ECD of which only one would be considered “properly folded” for the ECD of a class B GPCR (18, 42–46). To isolate the fraction of the monomer pool that is properly disulfide-bonded, we generated a ligand affinity column using a low affinity analogue of astressin (bAS-OH) as the ligand. The weak but specific binding interaction allowed for the separation of folded and misfolded trx-ECD using native buffer conditions as outlined in Fig. 2C. To validate this approach, we compared the relative CRF binding activities of the monomer pool of trx-ECD (load), trx-ECD that flows through in the void volume of the column (peak A), and trx-ECD that is transiently retained (peak B) using a blot overlay binding assay with biotinylated CRF as the probe (Fig. 2D). The results clearly show that there was no CRF binding activity in trx-ECD from peak A, although there was a significant increase in binding activity in trx-ECD from peak B relative to what was loaded onto the column. A qualitative measurement of the increase in specific binding activity can be taken from the fact that a 1/5th equivalent of trx-ECD from peak B has more CRF binding activity than the material loaded onto the column. Material from peak B, which has been reduced and carbamethylated, has no binding activity in the overlay assay.

Identical binding results were obtained using biotinylated astressin as the probe (not shown). Purified trx-ECD was soluble in aqueous buffer at concentrations up to 100 μM.

A more quantitative assessment of ECD binding was carried out using an SPA with radiolabeled astressin as the probe. $K_i$ values were obtained for astressin (17 nM), CRF (3.1 μM), cCRF$_{30-41}$ (545 nM), and YAM19 (5.9 nM) (SPA curves are provided in the Supplemental Material). The value for astressin is comparable with previously reported values against the isolated ECD of the CRF₁ receptor where astressin was used as the radioligand (18, 19). The $K_i$ values for astressin and CRF are an order of magnitude higher than those measured by Klose et al. (19); however, this is to be expected because urocortin I was used as the radioligand in their assay, and their results demonstrate that astressin has a significantly higher affinity for soluble ECD than urocortin I. Together, our binding data demonstrate...
that the recombinant protein produced for these studies is correctly folded.

**NMR Characterization of cCRF30–41 Bound to trx-ECD—**

The $^1$H and the $^{13}$C assignments for cCRF30–41 are reported in Table 1. Resolution of the six C-terminal residues was aided by the strategic $^{13}$C labeling within this span (see “Experimental Procedures”). The lack of chemical shift dispersion, HN-H coupling constants, and lack of long range ROEs indicated that cCRF30–41 was unstructured when alone in solution (35).

In transferred NOE experiments, information relating nuclei in the ligand is "transferred" from the bound state (slow tumbling, negative NOEs) to the free state of the ligand (intermediate tumbling, no NOEs). The transfer is effective if the dissociation constant (off-rate) of the ligand from the protein is faster than the longitudinal relaxation rate of the magnetization of the peptide ($T_1$) (30, 47). The affinity of cCRF30–41 for the ECD at neutral pH and room temperature did not yield significant transferred NOEs, presumably because of a slow off-rate of the peptide from the ECD. Therefore, pH and temperature were adjusted until the strongest trNOEs were observed, presumably by lowering the binding affinity of cCRF30–41 for the ECD. Optimal results were obtained for a pH range between 4.0 and 5.0 at 310 K (37 °C). At pH values below ~3.8, no binding of cCRF30–41 to the trx-ECD was observed via NMR or standard pharmacological assays.

The trNOE spectrum shown in Fig. 3B shows that, in comparison with the ROESY spectrum from Fig. 3A, the sign of the NOEs has changed. This indicates binding to the trx-ECD protein. In addition, a few new peaks have appeared, and the relative intensities of many peaks have changed. In the absence of the trx-ECD protein, the corresponding NOE spectrum contains no off-diagonal cross-peaks. To confirm the specificity of binding, the high affinity peptide YAM19 was added to a 25 μM concentration, and the trNOE cross-peaks disappeared (data not shown). In the ROESY spectrum of the free peptide, the locations of a few of the expected αβ(i,i + 3) correlations are circled, and their absence is indicative of an unfolded free conformation of the peptide. In the bound trNOE spectrum, however, it is clear that the αβ(i,i + 3) correlations are present and therefore that the peptide has become structured. No trNOEs were found to the C-terminal amide, indicating that it does not contribute to the peptide structure.

**FIGURE 3. Comparison of the ROESY spectrum of the peptide alone in solution (A) and the trNOE spectrum of the peptide in the presence of trx-ECD (B). The circled areas show that, although many of the expected correlations are heavily overlapped, several αβ(i,i + 3) correlations appear in the trNOE spectrum that are consistent with the α-helicity of the bound peptide.**

### Table 1

Chemical shift assignments ($^1$H and $^{13}$C) for 2 mM cCRF30–41 in 50 mM NaAc-d$_4$ buffer, pH 4.5 at 310 K

| Residue   | HN  | Hα | Hβ  | Other         | $^{13}$C |
|-----------|-----|----|-----|---------------|----------|
| H$_2$C    | 8.43| 4.09| 2.09/2.04| 2.08          |          |
| Glu$_{30}$| 8.45| 4.24| 1.29  | H$^+$ 2.35    |          |
| Ala$_{31}$| 8.02| 4.72| 3.34/3.17| H$^+$ 8.59; H$^+$ 7.27 | C$^3$ 11.072 |
| His$_{32}$| 7.80| 4.31| 2.87/2.80| H$^+$ 7.60; H$^+$ 6.93 | C$^3$ 31.00 |
| Lys$_{33}$| 8.43| 4.68| 1.77  | H$^+$ 1.47; H$^+$ 3.53/3.12; H$^+$ 7.95 | C$^3$ 48.89; C$^3$ 23.12; C$^3$ 19.46; C$^3$ 35.69 |
| Asn$_{34}$| 8.19| 4.35| 1.94/1.84| H$^+$ 1.69; H$^+$ 3.26; H$^+$ 7.21; H$^+$ 6.69 | C$^3$ 47.93; C$^3$ 34.60; C$^3$ 19.45; C$^3$ 15.89; C$^3$ 17.19 |
| Arg$_{35}$| 8.27| 4.27| 1.79  | H$^+$ 1.45; H$^+$ 1.70; H$^+$ 3.01 | C$^3$ 47.99; C$^3$ 25.17; C$^3$ 24.44; C$^3$ 9.30 |
| Lys$_{36}$| 8.09| 4.32| 1.63  | H$^+$ 1.63; H$^+$ 0.95/0.89 | C$^3$ 53.57; C$^3$ 31.00; C$^3$ 19.73; C$^3$ 9.81; C$^3$ 5.13 |
| Met$_{37}$| 8.16| 4.45| 2.59/2.16| H$^+$ 2.66/2.59; H$^+$ 2.11 | C$^3$ 52.28; C$^3$ 30.81; C$^3$ 19.67; C$^3$ 9.88; C$^3$ 5.16 |
| Glu$_{38}$| 8.17| 4.32| 2.14  | H$^+$ 2.33/2.29 |          |
| Ile$_{39}$| 8.11| 4.23| 1.96  | H$^+$ 0.96; H$_2^+$ 1.57/1.26; H$^+$ 0.93 |          |
| Ile$_{40}$| 8.11| 4.16| 1.86  | H$^+$ 0.94; H$_2^+$ 1.50/1.22; H$^+$ 0.87 |          |
| Lys$_{41}$| 8.23| 4.23| 1.96  | H$^+$ 2.27/2.22 |          |
| ~NH$_2$   |     |    |      | H$^+$ 5.71; H$_2^+$ 7.07 |          |
Using the selectively $^{13}$C-labeled peptides, a set of additional trNOE restraints were measured from a two-dimensional trNOE-HSQC experiment. This data set resolved three overlapped inter-residual distance restraints at the C terminus of the peptide. In the $^{13}$C-resolved data sets, a very weak NOE relating H$^\alpha$ of Asn$^{34}$ to H$^\beta$ of Leu$^{37}$ was found. In addition, $\alpha$$\beta$($i,i+3$) NOEs were measured relating Arg$^{35}$ to Met$^{38}$, as well as Leu$^{37}$ to Ile$^{40}$.

The number of NOEs obtained was not sufficient for us to determine a high resolution structure for cCRF$^{30-41}$. However, there were sufficient data to define an $\alpha$-helical model of cCRF$^{30-41}$ when bound the ECD. A summary of the statistics for the calculation of the model is given in Table 2, and the completeness of these trNOEs is shown schematically in Fig. 4.

**Binding Epitope of cCRF$^{30-41}$**

TABLE 2
Statistics for the trNOE restraints used in the model building for cCRF$^{30-41}$ bound to the trx-ECD of CRF$^1$

| No. of calculated structures | 50 |
|------------------------------|----|
| No. of accepted              | 30 |
| Average no. of NOE violations| 1.02|
| Total experimental restraints| 134|
| Intra-residual               | 89 |
| Sequential                   | 23 |
| Long range                   | 22 |

$^*$ r.m.s.d. applies to 20 lowest energy structures.

Corrected r.m.s.d. to mean structure

Backbone (Å) 0.72
Backbone and side chains (Å) 1.31

Ramachandran plot

Allowed region 92%
Generously allowed 8%

Using the selectively $^{13}$C-labeled peptides, a set of additional trNOE restraints were measured from a two-dimensional trNOE-HSQC experiment. This data set resolved three overlapped inter-residual distance restraints at the C terminus of the peptide. In the $^{13}$C-resolved data sets, a very weak NOE relating H$^\alpha$ of Asn$^{34}$ to H$^\beta$ of Leu$^{37}$ was found. In addition, $\alpha$$\beta$($i,i+3$) NOEs were measured relating Arg$^{35}$ to Met$^{38}$, as well as Leu$^{37}$ to Ile$^{40}$.

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**Binding Epitope of cCRF$^{30-41}$**

FIGURE 4. Intra- and inter-residue connectivities measured from 150-ms trNOE spectrum of 2 mM cCRF$^{30-41}$ with 40 $\mu$m trx-ECD in 50 mM NaAc-$d_4$ buffer, pH 4.5, at 310 K. Missing bars represent connectivities that were not assigned based on overlap of resonances but were treated differently from well resolved NOEs that were clearly absent.

trNOE spectrum of 2 mM cCRF$^{30-41}$ with 40 $\mu$m trx-ECD in 50 mM NaAc-$d_4$ buffer, pH 4.5, at 310 K.

| Ac | E$_{30}$ | A$_{31}$ | H$_{32}$ | K$_{33}$ | N$_{34}$ | R$_{35}$ | L$_{36}$ | K$_{37}$ | M$_{38}$ | E$_{39}$ | I$_{40}$ | i-$\alpha$-NH$_2$ |
|----|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|-------------|
| doN | d$i$N   | d$\alpha$($i,i+1$) | d$\beta$($i,i+1$) | d$\alpha$($i,i+2$) | d$\alpha$($i,i+3$) |

The one-dimensional $^1$H STD difference experiment is shown in Fig. 5 for a sample with a 100:1 excess of the peptide ligand over the trx-ECD, along with a control experiment in which the trx-ECD is not irradiated. In the downfield region of the spectrum, there are two peaks that remain in the difference spectrum. Two of these are assigned to the side chain amide of Asn$^{34}$ (7.61 and 6.93 ppm), and the second two are attributed to the C-terminal amide (7.57 and 7.07 ppm). Further upfield, three well resolved methyl groups are shown to have strong interactions with the trx-ECD. The peak at 2.11 ppm is attributed to the terminal $\epsilon$-methyl group of Met$^{38}$ and is the strongest of the three. The N-terminal methyl resonance of the peptide is at 2.08 ppm and is present but significantly less intense than the methionine methyl resonance. The peak at 2.02 ppm in the control experiment is attributed to residual nondeuterated acetate in the buffer and clearly has no interaction with the trx-ECD. The resonance at 1.295 is assigned to the H$^\beta$ methyl resonance of Ala$^{31}$ and is intermediate in intensity between the $\epsilon$-methyl Met$^{38}$ and N-terminal methyl interactions. Two-dimensional $^1$H/$^1$C STD HSQC experiments were performed using the two $^{13}$C-labeled peptides to further quantify the binding residues of the peptide in the region of the $^1$H NMR spectrum where the signals were less resolved.

For this 12-residue peptide, the strongest interactions are attributed to four groups: the terminal methyls of Ile$^{41}$, the terminal $\epsilon$-methyl of Met$^{38}$, the amide protons of Asn$^{34}$, and the C-terminal amide of the peptide. Three weaker interactions are attributed to the methyls from the N terminus, Leu$^{37}$ and Ala$^{31}$.

**Model of Bound cCRF$^{30-41}$**

The information presented here enables the construction of a model of the peptide and the epitope that defines its interaction with the ECD of the CRF$_1$ receptor. Fig. 6A shows a backbone overlay of the 20 lowest energy structures and displays good convergence, with a backbone root mean square deviation of 0.72 Å. It is interesting to note the slight unwinding of the helix at its C terminus; however, it is difficult to speculate on the significance of this fact because of the lack of a number of NOEs in this region of the peptide. The overlay shown in Fig. 6B demonstrates that the side chain conformations were well defined from the NOE data
for the residues involved in direct interactions with the receptor, with a total heavy atom root mean square deviation of 1.31 Å. The 13C labeling was a major contributing factor to the resolution of these resonances. The minimum energy structure shown in Fig. 6C illustrates the basic structural features of the helical peptide as well as the binding epitope. The residues involved in the binding interface are on one face of the helix. The presence of a potential salt-bridge interaction in the peptide on the backside of the peptide is evident, roughly 90° in pitch away from the covalent lactam constraint connecting residues Glu30 and Lys33. A three-dimensional view of the pharmacophore is shown in Fig. 6D. This view highlights the residues forming the most intimate and productive interactions with the ECD of the CRF1 receptor. This pharmacophore includes hydrophobic interactions and two amide functionalities, one at either end of the hydrophobic patch.

**DISCUSSION**

In the experimental results presented here, we outline a method for the expression and purification of milligram quantities of the correctly folded ECD of the CRF1 receptor from *E. coli*. We then apply this recombinant protein as a tool in structural studies of the peptide antagonist cCRF30–41 by NMR. The results of these NMR experiments revealed that cCRF30–41 exists as an unstructured peptide when free in solution. However, when bound to the ECD of the CRF1 receptor, this peptide becomes structured and adopts an α-helical conformation. The juxtaposition of residues Arg35 and Glu39 also suggested that a salt bridge may occur between these residues in the bound conformation of the peptide.

In addition to determining the bound structure of cCRF30–41, it was possible to map the residues that are in closest contact with the ECD and make up its binding epitope. These residues reside primarily on one face of the helix. The strongest interactions come from the hydrophobic residues Met38 and Ile41 and from two amide functionalities, Asn34 and the C-terminal amide. Weaker interactions were present with the side chains of Ala31 and Leu37. It is important to note that even though cCRF30–41 contains an unnatural helix-stabilizing lactam ring, which enhances its potency as an antagonist, the binding epitope itself is made up of amino acids and the C-terminal amidation which occur naturally in CRF. Substitutions that increase the hydrophobicity of the peptide at positions 31 and 38 underlie the significantly higher affinity of the more potent peptides of identical size (see peptides 19 and 20) when compared with cCRF30–41, supporting the role of these positions in the interaction of CRF with the ECD (26). In fact, the map of the binding epitope highlights the importance of the residues common to CRF, astressin, sauvagine, urocortins I, II, and III, and the truncated series of peptide antagonists from Rijkers *et al.* (25) and Yamada *et al.* (26). (The amino acid sequences of the larger family of peptide ligands are given in the Supplemental Material.) In all of the peptides, the Asn34 residue is absolutely conserved, and the C-terminal amide has also been found to be essential for the potency of these peptides. Likewise, comparison of these sequences demonstrated the invariance of a large hydrophobic residue at positions 38 and 41. The residues Ala31, Arg35, Leu37, and the acidic residue at position 39 are also present in all but a few peptide sequences. Overall, we find that our data agree well with the binding interface originally proposed by Yamada *et al.* (26), with the exception of the fact that the binding epitope seems to be rotated one residue counter-clockwise on the helical wheel. One potential interaction that was not seen in these
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studies was from His³². Given that the pKₐ of His residues is 6.1, this is the only residue whose charge is affected by the change in pH that was necessary for the NMR experiments. Therefore, we cannot rule out that this residue could play a role in the interaction with the ECD at neutral pH.

There is one potential discrepancy between our data and a previously published mutagenesis study. Mutations of Arg⁵⁵ to an Ala residue were not tolerated in either a minimal peptide or full-length ovine CRF (25, 49), yet another report demonstrated that mutations of that residue to Ala or Lys were tolerated at that position (26). Our experiments indicate the absence of a direct interaction of the ECD with Arg⁵⁵. As can be seen from the side view of the peptide structure in Fig. 6C, Arg¹⁵ and Glu⁹⁹ are in close proximity. The conservation of Arg⁵⁵ and the deleterious effect of mutations of this residue, taken together with the conservation of an acidic residue at position 39, suggest a helix-stabilizing (i, i + 4) salt bridge. Mutagenesis studies of ovine CRF showed that Ala substitution of the acidic residue at position 39 was tolerated; however, Ala is a helix-stabilizing residue, and its presence may abrogate the need for a stabilizing salt bridge.

The molecular details of the interaction between the CRF₁ receptor and its ligands were recently enlightened by the report of an NMR structure for the highly homologous ECD of the mouse CRF₂(b) receptor (50). Their results are relevant because of the sequence conservation between the mouse CRF₂(b) and the human CRF₁ receptors. Likewise, the peptide antagonists astressin and the cCRF₃₀–₄₁ have a large degree of sequence similarity. Although it was clear from their results that there is a significant structural rearrangement upon binding, the authors were able to localize the binding residues to one particular location on the ECD. Twelve of the 14 residues on the ECD are identical between the two isoforms of the receptor. The residues implicated outline a hydrophobic patch on the ECD and contain several opportunities for productive hydrogen bonding (donors and acceptors) with the peptide ligand. This surface includes three hydroxyl-bearing side chains, an asparagine, two positive charges, one negative charge, and seven hydrophobic residues. The few charged residues involved are peripheral to the main binding location on the ECD and had smaller chemical shift perturbations. This is complementary to the results presented here with regard to the fact that the residues responsible for this interface are localized to particular residues on the peptide ligand.

Using the combination of these data, attempts were made to construct a model of this minimal peptide antagonist bound to the human CRF₁ ECD. Two factors prohibited the construction of such a model. First, the data presented in the study by Grace et al. (50) suggest that there is a large scale conformational change in the ECD upon ligand binding. There is no structural data currently available for this change. Second, although we have specific structural data for the ligands and ECD based on mutagenesis and/or NMR data, there are no complementary data that allow for the positioning of any specific interactions between the peptide ligand and the ECD. Such information has been obtained in the past through photoaffinity labeling for the ECD-peptide ligand interaction of the VPAC-1 and PACAP receptors. However, there is not sufficient sequence similarity with these receptors to aid in building such a model.

Although a vast amount of indirect data has suggested that class B GPCR peptide ligands are helical in nature, only one other study has directly addressed ligand structure in the presence of its receptor. Inooka et al. examined the structure of a C-terminally truncated version of PACAP while bound to full-length receptor (51). This study focused on the interaction of the central region of the ligand presumed to interact with the ECD and the N terminus that interacts with the receptor heptahelical domain and activates the receptor. The key difference between these studies is that they examined the structure of a full agonist lacking the C terminus, whereas we examined a pure competitive antagonist with identity to only the C terminus of the full-length ligand. Assuming that there are conserved features for ligand-receptor interactions between the class B GPCR family, the two studies may be considered complementary in that they examine the structure of different parts of the full-length peptide agonists.

The data obtained from NMR spectroscopy in these studies is significant for two reasons. First, the three-dimensional structure of the peptide while bound to the ECD of its receptor was modeled. Second, the residues of the peptide responsible for direct binding to the ECD were determined. This information was transformed into a pharmacophore model that may be used to develop better minimal peptide and small molecule antagonists. Previous classes of small molecule antagonists for CRF₁ receptors have been shown to behave effectively as inverse agonists, binding to the active site and inhibiting activation even in constructs of the receptor that lack the ECD (14). For the CRF₁ receptor and its interaction with this minimal peptide antagonist, the information presented here represents an alternative strategy to the one previously employed (14, 52).

To date, all of the non-peptide small molecule CRF₁ receptor antagonists have targeted the juxtamembrane portion of the receptor and act allosterically to inhibit the function of the agonist peptides (53–55). The precise positioning of hydrophobic interactions and hydrogen bond donors and acceptors provides a starting point for the design of purely competitive small molecule antagonists. The pharmacophore, when measured end to end, spans ~15 Å and is a bit large. A novel small molecule would therefore presumably occupy only a subset of these interaction sites. In the future, a three-dimensional structure of the peptide ligand bound to this receptor would provide the information necessary to design optimal small molecule antagonists. The discovery of small molecules that could interact directly with the N terminus of the CRF₁ receptor and thus block the initial event of agonist peptide binding would represent a novel approach to inhibiting the function of this receptor and would offer a new class of potential therapeutics for stress-related and mood disorders.

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