A Role for a Helical Connector between Two Receptor Binding Sites of a Long-chain Peptide Hormone*

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The conformational freedom of single-chain peptide hormones, such as the 41-amino acid hormone corticotropin releasing factor (CRF), is a major obstacle to the determination of their biologically relevant conformation, and thus hampers insights into the mechanism of ligand-receptor interaction. Since N- and C-terminal truncations of CRF lead to loss of biological activity, it has been thought that almost the entire peptide is essential for receptor activation. Here we show the existence of two segregated receptor binding sites at the N and C termini of CRF, connection of which is essential for receptor binding and activation. Connection of the two binding sites by highly flexible ε-aminocaproic acid residues resulted in CRF analogues that remained fully active, although weak agonists (EC50 100–300 nM) independent of linker length. Connection of the two sites by an appropriate helical peptide led to a very potent analogue, which adopted, in contrast to CRF itself, a stable, monomeric conformation in aqueous solution. Analogues in which the two sites were connected by helical linkers of different lengths were potent agonists; their significantly different biopotencies (EC50 0.6–50 nM), however, suggest the relative orientation between the two binding sites rather than the maintenance of a distinct distance between them to be essential for a high potency.

The biologically important peptide hormones corticotropin releasing factor (CRF)1, glucagon, secretin, vasoactive intestinal polypeptide, growth hormone releasing factor (GRF), calcitonin, parathyroid hormone (PTH), calcitonin gene-related peptide (CGRP), etc. have significant features in common. All exert their activity via binding to and activation of class 2 G protein-coupled receptors (GPCRs). They are polypeptides comprising about 25–40 amino acid residues without preferred conformation in aqueous solution and exhibit no documented biologically relevant secondary and tertiary structure. Under structure-inducing conditions (e.g. in the presence of trifluoroethanol or membrane mimicking lipids), however, these peptide hormones (CRF (Ref. 1), PTH (Refs. 2 and 3), calcitonin (Ref. 4), glucagon (Ref. 5), GRF (Ref. 6)) exhibit strong helix formation, suggesting that a helical ligand conformation is essential for receptor interaction. Furthermore, N-terminal truncations of the hormones convert them from agonists to antagonists (CRF (Ref. 7), GRF (Ref. 8), calcitonin (Refs. 9 and 19), PTH (Ref. 11), CGRP (Ref. 12), glucagon (Ref. 13)), proving the existence of an important receptor binding/activation site within the peptide N terminus. C-terminal truncations result in a drastic decrease in receptor binding, indicating an essential function for C-terminal residues (CRF (Refs. 9 and 14), GRF (Ref. 15), CGRP (Ref. 16)). Based on these results, it has been assumed that almost the entire peptide is necessary for binding to and activation of the corresponding receptors.

In the case of CRF, which is the principal neuroregulator of the basal and stress-induced secretion of ACTH, β-endorphin and other proopiomelanocortin-related peptides from the anterior pituitary (see Ref. 17 for review), previously published structure-activity relationship studies of single-point substituted (18–20) and terminally truncated CRF analogues (7, 21) showed that the N-terminal sequence (9–19) represents a receptor binding site, since substitutions in this region resulted in a significant decrease in receptor binding. The most N-terminal amino acid residues are thought to be responsible for receptor activation (7), since truncation of these residues produced antagonists. The N-terminal peptide sequence (6–20) is highly conserved within the CRF family, peptides from different species that activate CRF receptors. In contrast, there is great sequence diversity within the C-terminal region (21–41). Substitutions of Arg-35 or Leu-38 in oCRF by alanine (18), conversion of the C-terminal carboxamide to a carboxyl group or truncation of the C-terminal dipeptide from oCRF, however, reduced biopotency dramatically (14), indicating an essential binding site to be located at the extreme of the C terminus. The existence of two receptor binding sites in peptide ligands of class 2 GPCRs was also suggested by studies using chimeric receptors and peptide ligands, but nothing has been described concerning the structural organization of the ligands (22, 23).

We have investigated whether segregated receptor binding sites in CRF do exist and, if so, what role the connector unit between the two binding sites might play. CRF analogues with highly flexible, structurally simplified as well as conformationally stabilized connector units between the two sites were investigated to address these questions.

**EXPERIMENTAL PROCEDURES**

**Preparation of Peptides**—Peptides were synthesized automatically (MilliGen 9050 peptide synthesizer) by the solid-phase method using standard Fmoc (N'-9-fluorenylmethoxycarbonyl) chemistry in the continuous flow mode as described previously for the synthesis of CRF analogs (20). Purification was carried out by preparative HPLC to give final products of >95% purity by reverse phase-HPLC analysis. The peptides were characterized by mass spectrometry, which gave the...
expected [M+H]⁺ mass peaks and correct amino acid analyses.

**Peptide-Produced Testosterone Production**—Leydig cells were prepared from adult, male NMRI mice as described previously (24) and allowed to attach to well plates (100,000 cells). Medium was removed and replaced with 1 ml of fresh incubation medium containing the phosphodiesterase inhibitor IBMX (2.5 μM) and CRF or CRF analogues (0.01 nM to 1 μM). Incubations were performed in a shaking water bath (35 rpm) at 37 °C for 30 and 60 min. 100 μl of the medium were frozen for the determination of testosterone by radioimmunoassay (DPC Bier-

**ACTH Releasing Activity of Peptides on Rat Anterior Pituitary**

| Peptide                | EC₅₀ Ref. |
|------------------------|-----------|
| Ovine CRF (oCRF)       | nanomolar |
| SQEPPISLDLTFHLLREVMETKADQIAQQASHNRKLLLDIA-NH₂ | 4.82 (24) |
| Cerebellomedullin      | nanomolar |
| NDD----I--------NMI----ARNENQRE--G---Y--E--V-NH₂ | 2.67 (24) |
| Frog sauvagine         | nanomolar |
| <G-E----SLE--------KMI--IEKQJEKEKQ---AN---L---TI-NH₂ | 2.14 (24) |
| Rat Uct                | nanomolar |
| DD----L--I--------TLL--LARTQSQER--EQ----III--F--S--V--NH₂ | 0.79 (24) |
| h/rCRF                 | nanomolar |
| --E--------------------AR--E--------G---Y--E--V--NH₂ | 4.15 |
| Chimeric               | nanomolar |
| oCRF(1-20)-UtS(21-41)  | 3.85 |
| oCRF(1-20)-SvG(20-40)  | 3.85 |
| UtS(1-20)-SvG(20-40)   | 3.85 |
| NDD----I--------NMI--IEKQJEKEKQ---AN---L---TI-NH₂ | 1.03 |

The Table I shows the EC₅₀ values for the determination of ACTH. The EC₅₀ values were calculated from the dose-response curves by a four-parameter logistic curve-fitting program.

**CD Spectroscopy**—CD measurements were carried out on a Jasco 720 spectrometer from 185 to 260 nm. The amount of helix was estimated from the relation: \( \% \text{helix} = \left( \theta^s_222 - \theta^s_322 \right) / \left( \theta^h_222 \right) \), where \( \theta^s_222 \) is the determined mean residue ellipticity at 222 nm. For \( \theta^s_222 \) and \( \theta^h_222 \), representing 0 and 100% helix content, values of 2340 and 30,300 degrees-cm²/dmol, respectively, were used (28). For determina-

**Structural Simplification of CRF**—Intramolecular interactions between the N and C termini of long-chain peptide ligands, e.g., CRF (31), have been suggested as stabilizing a
biologically active conformation. Therefore, amino acid variations within the N and C termini (non-conserved amino acid residues) within the CRF family may function interdependently in such interactions. In order to address the question, we synthesized chimeric peptides, combining the N and C termini of oCRF, urotensine, and sauvgaine. The chimeric peptides exhibited a high biological potency (Table I), showing that these amino acid variations are, in fact, not interdependent with respect to stabilization of the biologically active conformation.

Assuming an \( \alpha \)-helical conformation of CRF to be advantageous for receptor interaction, substitution of those amino acid residues of CRF that are not individually essential for receptor interaction, especially the non-conserved residues within the CRF family, by others with a high helical propensity, such as alanine, should be possible without loss of biopotency. Thus, while retaining arginine-35 and the hydrophobic residues at positions 36/37/38 (leucine), the remaining amino acid residues within the C-terminal portion (residues 22–41) of h/rCRF were replaced by alanine or glutamine residues. This modification yielded an analogue of high biological potency (Table II), demonstrating that the amino acid residues of the middle portion within the C-terminal portion (residues 22–33) of CRF are not individually essential for receptor interaction. From these results, the question arose as to whether the middle portion is essential at all for receptor interaction, especially the non-conserved residues within the CRF family. Therefore, amino acid variations are, in fact, not interdependent with respect to stabilization of the biologically active conformation.

TABLE II

Primary structure and biological potency (testosterone production) of simplified CRF analogues

| Peptide                        | EC\textsubscript{50} (nM) |
|--------------------------------|--------------------------|
| h/rCRF                         | 2.84                     |
| SQEPPISLDLTFHLLREVLEMARAEQLAQHASNRLKMLE1-NH\textsubscript{2} |                       |
| Poly-alanine-substituted h/rCRF| 3.35                     |
| SQEPPISLDLTFHLLREVLEMARAAQAQAQAAANRLLLAAANH\textsubscript{2} |                       |
| Direct connection of the receptor binding sites (acp\textsubscript{1}) | 175                      |
| DDPPLSIDLTFHLLRTLLEIEACPNRKLLDEV-V-NH\textsubscript{2} |                       |
| Connection of the receptor binding sites by \( \epsilon \)-aminocaproic acid residues |                       |
| acp\textsubscript{1}          | 77                       |
| DDPPLSIDLTFHLLRTLLEIEACPNRKLLDEV-V-NH\textsubscript{2} |                       |
| acp\textsubscript{2}          | 200                      |
| DDPPLSIDLTFHLLRTLLEIEACPACPACPNRKLLDEV-V-NH\textsubscript{2} |                       |
| acp\textsubscript{3}          | 300                      |
| DDPPLSIDLTFHLLRTLLEIEACPACPACPACPACPNRKLLDEV-V-NH\textsubscript{2} |                       |
| acp\textsubscript{4}          | >10\textsuperscript{4}  |
| N-terminal binding site        |                          |
| DDPPLSIDLTFHLLRTLLEIEACPACPACPACPACPACPACPACPACPACPACPACPVR-NH\textsubscript{2} |                       |
| C-terminal binding site        |                          |
| NRKLLDEV-V-NH\textsubscript{2} | >10\textsuperscript{4}  |

FIG. 1. Effect of urocortin (uct), oCRF, and a CRF analogue in which the two receptor binding sites were connected by an \( \epsilon \)-aminocaproic acid residue (acp\textsubscript{1}) on testosterone production in mouse Leydig cells.

**Role of Connector between Receptor Binding Sites**

**Potentially \( \alpha \)-Helical Connector Units between the Two Receptor Binding Sites**—Circular dichroism (CD) spectroscopy studies of the potent polyalanine-substituted h/rCRF agonist in aqueous solution revealed strong helix induction by alanine incorporation, indicating that the N- and C-terminal binding sites of CRF might be advantageously connected by an \( \alpha \)-helical structure. The helical content of the analogue was found to be concentration-dependent, i.e. the formation of secondary structure is at least partially caused by association (data not shown). In order to prevent such association, we created an analogue in which the two binding sites were connected by a highly charged peptide consisting exclusively of glutamic acid and lysine residues arranged such that helix stabilization could occur by salt bridge formation (32) between side chains at positions \( i \) and \( i + 4 \) (EKEEKEKKRKE). The resulting analogue, urocortin-\( \text{EK} \) (UEK), was found to be at least as potent as the most active member of the CRF family, urocortin (Table III). The result was confirmed in an \( \text{in vitro} \) pituitary cell assay for peptide induced ACTH release (EC\textsubscript{50} for Uct, 0.06 nM, and for UEK, 0.04 nM). CD studies revealed an \( \alpha \)-helical content for UEK of 45% at 4 °C in aqueous solution, independent of concentration (1 \( \mu \text{M} \) to 1 mM), indicating the acquisition of a monomer conformation. The existence of a monomer solution in water at a concentration of 1 mM was confirmed by light-scattering studies (data not shown). To demonstrate that the contribution of individual amino acid residues of the EK-connector is not re-
sponsible for the high biopotency of UEK, we substituted the EK-connector (residues 22–31) in UEK by the alanine-rich sequence RAAAQAAKKA. The corresponding analogue was at least as potent as UEK itself (Table III).

In order to investigate whether the length of the connector unit (22–31) in UEK plays a specific role with respect to biopotency, we synthesized the corresponding deletion analogues. Stepwise shortening of the connector unit of UEK by deletion of one (des(23)-UEK, des(25)-UEK, des(26)-UEK, des(28)-UEK, des(29)-UEK), two (des(23, 24)-UEK), three (des(23–25)-UEK), four (des(23–26)-UEK), five (des(23–27)-UEK), six (des(23–28)-UEK), or seven (des(23–29)-UEK) amino acid residues, respectively, had very different effects on biological potencies compared with UEK (Table III). The deletion of five amino acid residues led to a drastic decrease in biopotency of about 2 orders of magnitude, but the deletion of seven residues resulted in a relatively minor loss (Table III). Moreover, a corresponding deletion of three amino acid residues led again to a high biopotency comparable to that of UEK itself. Furthermore, elongation of the EK connector in UEK by seven amino residues (UEK-t5) resulted in a significant decrease in biopotency (Table III), possibly due to there being too great a distance between the two sites. Thus, in contrast to the acp analogues, which showed a similar biopotency while bearing different numbers of acp residues in the linker between the two receptor binding sites, the variation of the connector length in UEK strongly affected biopotency. CD measurements of the analogues (50 mM) in aqueous buffer solution (pH 7.1) at room temperature revealed that the acp analogues possess no preferred conformation (Fig. 2), while the shortened UEK-analogues revealed a significant helical conformation (des(23–25): 36%, des(23–26): 23%, des(23–27): 38%, des(23–28): 21%, des(23–29): 36%). After deletion of 14 amino acid residues of the central part of UEK, des(19–32)-UEK, no preferred conformation was observed, in contrast to UEK itself which revealed under these conditions an α-helical content of 39% compared with 34% at pH 3.4. Thus, linkers that consisted of α-amino acid residues as in UEK and its centrally shortened analogues induced and/or stabilized an α-helical ligand conformation whereby the different lengths of the linkers, in contrast to highly flexible acp connector units, showed a strong effect on receptor activation. To investigate the effect of single amino acid deletions in the suggested connector unit of the wild-type ligand oCRF on biopotency, we synthesized des-Thr(22)-oCRF and des-Gln(29)-oCRF.

### Table III

| Linker | Primary structure and biological potency (testosterone production) of UEK analogues (peptide amides) |
|--------|------------------------------------------------------------------------------------------------|
|        | EC_{50} | nM | (net charge) |
| UEK    | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 0.6 | +1 |
| Dex (23) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 25 | +2 |
| Dex (25) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 27 | 0 |
| Dex (26) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 13 | +2 |
| Dex (28) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 17 | 0 |
| Dex (29) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 50 | 0 |
| Dex (23, 24) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 8.5 | +3 |
| Dex (23–25) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 0.9 | +2 |
| Dex (23–26) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 12 | +3 |
| Dex (23–27) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 50 | +2 |
| Dex (23–28) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 30 | +1 |
| Dex (23–29) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 4.6 | 0 |
| RAAAQAAKKA (22–31) - UEK | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 0.4 | +2 |
| UEK-t5 | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 1,700 | 0 |
| UEK (1–38) | D D P P L S I D L T F H L L R T L L D E I E K K E K E K K K K K R K K K E Q N R K L L D E | 140 | +1 |

![CD spectra of urocortin-EK (UEK), des(23–29)-UEK and acp1 (0.05 mM) in aqueous buffer (24 °C, pH 7.1).](fig2.png)
both analogues exhibited reduced biopotencies (des-Thr(22)-oCRF (EC$_{50}$: 146 nM), des-Gln(29)-oCRF (EC$_{50}$: 57 nM)) compared with oCRF (EC$_{50}$: 3.82 nM) as determined by the testosterone assay, a result which is similar to that obtained for corresponding deletion analogues of UEK.

Assuming a two-site receptor binding mode in the case of UEK and oCRF, corresponding amino acid deletions in the suggested binding sites should strongly affect receptor interaction. Consistently, the truncation of UEK by the two C-terminal amino acid residues, resulting in UEK (1–38)-amide, led to a drastic decrease in biopotency (Table III), showing the C-terminal binding site to be much more sensitive than the connector unit toward deletion/truncation with respect to biopotency. Shortened analogues of the wild-type ligand oCRF des-Asp(39)-oCRF (EC$_{50}$: > 1 µM) or des-Thr(11)-oCRF and des-Glu (17)-oCRF (both were found to be inactive at a peptide concentration of 1 µM)) showed again strongly reduced biopotencies deleting single amino acid residues of the binding sites as determined by the testosterone assay.

In order to strengthen the indication that an α-helical linker connects the two receptor binding sites in the biologically very potent UEK, we investigated the structure of UEK in aqueous solution by $^1$H NMR spectroscopy.

Solution Structure of UEK Determined by Two-dimensional NMR Spectroscopy and Molecular Modeling—The $^1$H NMR assignments of UEK were made using standard procedures (33) and the main chain-directed approach (34) on the basis of double-quantum filtered DQF-COSY, TOCSY, and NOESY spectra at 5 °C in 90% H$_2$O, 10% D$_2$O. In the amide region of the two-dimensional NOESY spectrum shown in Fig. 3A, nearly all sequential NH(i)-NH(i+1) cross-peaks between Thr-10 and Lys-30 were resolved. Extensive unambiguous medium-range NOEs αN(i,i+3) and αδ(i,i+3) NOEs, characteristic of an α-helical conformation, were observed for UEK 10–30 and confirmed helix formation, at least from Thr-10 to Lys-30. Besides NOEs, the chemical shifts for Ca-protons (35) are commonly used for secondary structure assignments in peptides. In particular, the chemical shifts of Ca-protons within α-helices or β-sheets tend to exhibit an upfield or downfield shift respectively, relative to the chemical shift values typical for a random-coil conformation. The plot of the Ca-proton chemical shift deviations versus the position along the sequence of UEK is shown in Fig. 4. Applying the criteria for secondary structure, the differences between observed and random-coil Ca proton chemical shifts from Thr-10 to Lys-35 show negative values, consistent with an α-helical arrangement. In addition to the helical domain 10–30, a limited number of long-range NOEs suggest a preferred loop conformation. The plot of the Ca-proton chemical shift deviations versus the position along the sequence of UEK is shown in Fig. 4. Applying the criteria for secondary structure, the differences between observed and random-coil Ca proton chemical shifts from Thr-10 to Lys-35 show negative values, consistent with an α-helical arrangement.

A three-dimensional structure of UEK was calculated following the molecular dynamic protocol described under “Experimental Procedures” using a final set of 268 NOE-derived distance constraints (113 intra, 93 sequential, 59 medium-range, and 3 long-range > 5 residues) at 5 °C. Because of peak overlapping, NOE intensities were not classified. After minimization 10 structures, based on low residual distance violations and low dihedral angle violations, were selected and used to compute the solution structure of UEK. Fig. 3B displays the 10 final structures superimposed for the minimum backbone deviations between residues 10 and 30, 1 and 9, and 31 and 40. Whereas an α-helix is well defined in the central part of UEK between residues Thr-10 and Lys 30, the N and C termini themselves exhibit some evidence of local order. The central helix comprises the N-terminal binding site (Fig. 3C, violet) and an α-helical connector (Fig. 3C, green) to the C-terminal binding site, i.e. an α-helix of about 3 turns forms the linker between the two sites.

**DISCUSSION**

The structural requirements of linear, long-chain peptide ligands for receptor interaction are more poorly understood than those of small molecules because of their numerous potential receptor interaction sites and great conformational freedom, which disfavors a stable conformation in water, a general obstacle to the determination of their biologically relevant conformation. The fact that N- and C-terminal truncations of various long-chain peptide ligands of class 2 GPCRs lead to a loss of biopotency had suggested that almost the entire peptide is essential for receptor activation.

We demonstrated here that stepwise structural simplification of a peptide ligand of this receptor class is a powerful approach to gain insight into the structural requirements of a peptide ligand for its receptor interaction. Multiple substitution of amino acid residues by alanine indicated that the amino acid residues of the middle portion (22–33) of CRF are individually not essential for receptor activation. Indeed, deletion of the middle portion led to a full agonist at nanomolar concentrations (apc0, Fig. 5). Substitution of this middle portion by 1, 2, 3, or 4 apc residues resulted in analogues which exhibited a similar biopotency (Table II, Fig. 5). Therefore, in the case of flexible apc linkers the linker length between the two receptor binding sites had only little effect on receptor activation. In contrast, truncation of the C-terminal residues (34–40) in the apc4 analogue led to a total loss of intrinsic activity, consistent with the existence of a second segregated receptor binding site in the peptide C terminus. Furthermore, the peptides that represent the two receptor binding sites, either alone or as an equimolar mixture, exhibited no receptor activation, demonstrating that connection of the two is essential for biopotency.

Because of the high helix forming propensity of alanine, the fact that an alanine-rich connector unit between the two binding sites led to an analogue showing a significantly increased biopotency compared with the corresponding apc analogue suggested an α-helical connector to be more appropriate for receptor interaction with the two sites. Alanine-rich peptides tend, however, to self-associate which hampers determination of their monomer solution conformation. On the contrary, the EK model peptide has been shown to be well suited not only to incorporate a stable α-helix in the peptide, but also to prevent self-association at millimolar concentrations, as observed with the native peptide ligands of CRF receptors. Therefore, incorporation of the highly charged EK helix allowed determination of the monomer structure of a biologically very active analogue (UEK) in water by means of NMR analysis. Although the solution structure of UEK was not totally resolved, the results clearly show the existence of a central α-helix (10–30) comprising the amino acid residues of the N-terminal receptor binding site and the connector unit to the C-terminal site. Incorporation of the stable EK helix led to an exact distance between both binding sites in the ligand and, thereby, a high biopotency, in contrast to the apc analogues with highly flexible linkers, which exhibited a drastic loss of biopotency compared with that of UEK. On the other hand, this shows that an appropriate distance between both binding sites in UEK, although advantageous, is not essential for receptor binding and activation. Deletions of an increasing number of amino acid residues from the connector helix in UEK led to an interesting pattern with respect to biopotency of the analogues. While the deletions...
resulted generally in a decrease in biopotency compared with that of UEK, a relatively minor decrease was observed by deletion of either three or seven amino acid residues. There seems to be no correlation between biopotencies of the deletion analogues and net charge of linker units or position of individual amino acid residues in the connector to explain the differ-
ent biopotencies (Table III). Assuming flexible connector units between the two receptor binding sites in the deletion analogues of UEK, they should exhibit a similar biopotency as in the case of the acp analogues. However, CD investigation of the deletion analogues of UEK revealed, in contrast to the acp analogues, a significant \( \alpha \)-helical content when the middle part (20–32) was not completely deleted, suggesting \( \alpha \)-helical connector units in the UEK analogues. In this case, the number of amino acid residues in the connector helix will determine not only the distance between the two receptor binding sites but also their relative orientation (Fig. 5). Since the stepwise shortening of the UEK connector helix did not directly correspond with biopotency of the analogues, the relative orientation of the two binding sites rather than the maintenance of a distinct distance between them seems to be essential for a high potency. A high biopotency of analogues bearing a helical connector may also be explained by induction and/or stabilization of an appropriate conformation of one or both receptor binding sites. The fact, however, that \( \text{des}(23–27)\)-UEK revealed an \( \alpha \)-helical content, which was at least comparable to that of UEK, but UEK is 80-fold more potent than \( \text{des}(23–27)\)-UEK, suggests that this conformational stabilization of the binding sites may play a minor role.

Comparing the very potent agonists UEK, \( \text{des}(23–25)\)-UEK and \( \text{des}(23–29)\)-UEK (Fig. 5), the corresponding distances between the two sites may be very different. Regarding receptor activation, biopotencies decreased in the order UEK (EC\(_{50}\): 0.6 nM) > \( \text{des}(23–25)\)-UEK (0.9 nM) > \( \text{des}(23–29)\)-UEK (4.6 nM). With respect to receptor binding, the same order was observed (UEK (\( K_d \): 0.4 nM) > \( \text{des}(23–25)\)-UEK (4.1 nM) > \( \text{des}(23–29)\)-UEK (8.1 nM)), indicating that the appropriate distance of the two sites in UEK is responsible for high receptor affinity and, thereby, for high biological potency. Furthermore, an analogue in which the two sites are connected directly via Ile-Glu-Gln (acp0) still exhibited full intrinsic potency (Fig. 5). Assuming that the analogues bearing the identical receptor binding sites bind to the same sites of the receptor, it appears that the distance between the hormone binding sites of the receptor may vary remarkably (by approximately 7 Å/helix turn). This large difference requires a remarkable flexibility of the receptor domains for ligand binding, which may be explained if one binding site of the ligand binds to the receptor N terminus and the other site to the remaining C-terminal portion of the receptor. For chimeric constructs of CRF and GRF receptors, it was shown that the extracellular domains, particularly the N terminus and the third loop of the CRF receptor (CRFR-1), are responsible for high affinity for urocortin (36).

In the case of the members of the CRF family, which do not adopt a preferred conformation in water, helix formation may result from interaction with the receptor or its surroundings.
Under structure-inducing conditions, CRF was shown to adopt a largely α-helical conformation, particularly in the middle part of the molecule. NMR spectroscopic investigations confirmed that oCRF and h/oCRF comprise a well defined α-helix between residues 6–36 in a mixed solvent system (TFE/H₂O) (1, 37). Both peptides bind to detergent micelles associated with an increase in the α-helical content (37) and, on interacting with hydrophobic interfaces, oCRF revealed an α-helical conformation comprising residues 6–32 (38). Furthermore, connector units between the two receptor binding sites consisting of amino acid residues with a high helical propensity resulted in a very potent agonist (Table II), and it was recently reported that incorporation of two adjacent D-amino acid residues, which causes a significant destabilization of α-helices (39), in the connector region of oCRF led to a drastic loss of biopotency (24). Moreover, single-point amino acid deletions in the corresponding connector unit of oCRF at positions 22 or 29 led to a decrease in biopotency which is comparable to the effect of corresponding deletions in UEK, while amino acid deletions in the suggested receptor binding domains of oCRF resulted in almost complete loss of biopotency. Therefore, it seems very likely that our results for UEK may be relevant also in the case of the wild-type ligands of the CRF receptor.

Similarly to UEK, the only peptide ligand of class 2 GPCRs that has a preferred structure in water, the 35-amino acid -helodermin possesses a stable central that has a preferred structure in water, the 35-amino acid residues 6–36 in a mixed solvent system (TFE/H₂O) (1, 37).

Interactions.

hormones which will also stimulate studies on ligand-receptor units to give biologically fully active analogues, opens a way for segregated receptor binding sites in a long-chain peptide ligand termini of such peptide ligands. Proof of the existence of two fined linker between receptor binding sites at the N and C portions of class 2 GPCRs, which can be linked by different connector

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A Role for a Helical Connector between Two Receptor Binding Sites of a Long-chain Peptide Hormone

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