Hexa-histidin tag position influences disulfide structure but not binding behavior of in vitro folded N-terminal domain of rat corticotropin-releasing factor receptor type 2a

JANA KLOSE,[1] NORBERT WENDT,[1] SYBILLE KUBALD,[1] EBERHARD KRAUSE,[1] KLAUS FECHNER,[1] MICHAEL BEYERMANN,[1] MICHAEL BIENERT,[1] RAINER RUDOLPH,[2] AND SVEN ROTHEMUND[3]

[1]Research Institute of Molecular Pharmacology, D-13125 Berlin, Germany
[2]Martin-Luther University Halle-Wittenberg, D-01620 Halle, Germany
[3]Interdisciplinary Center for Clinical Research, D-04103 Leipzig, Germany

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Abstract

The oxidative folding, particularly the arrangement of disulfide bonds of recombinant extracellular N-terminal domains of the corticotropin-releasing factor receptor type 2a bearing five cysteines (C2 to C6), was investigated. Depending on the position of a His-tag, two types of disulfide patterns were found. In the case of an N-terminal His-tag, the disulfide bonds C2–C3 and C4–C6 were found, leaving C5 free, whereas the C-terminal position of the His-tag led to the disulfide pattern C2–C5 and C4–C6, and leaving C3 free. The latter pattern is consistent with the disulfide arrangement of the extracellular N-terminal domain of the corticotropin-releasing factor (CRF) receptor type 1, which has six cysteines (C1 to C6) and in which C1 is paired with C3. However, binding data of the two differently disulfide-bridged domains show no significant differences in binding affinities to selected ligands, indicating the importance of the C-terminal portion of the N-terminal receptor domains, particularly the disulfide bond C4–C6 for ligand binding.

Keywords: protein folding; affinity tag; corticotropin-releasing factor receptor

Affinity tags of fusion proteins are widely used in recombinant protein expression techniques. The most common affinity tags, such as poly-histidine (Bornhorst and Falke 2000), glutathione S-transferase (GST; Smith 2000), maltose binding protein (MBP; Sachdev and Chirgwin 2000), and streptavidin tags (Skerra and Schmidt 2000), are routinely used for increasing expression, enhancement of solubility, and protein purification. In novel developments of high-throughput screening techniques, such as scintillation proximity assay (SPA) and alphaScreen (PerkinElmer Life and Analytical Sciences, Inc.), one or two proteins, respectively, are attached to beads via affinity tags. It is known that large affinity tags (MBP, GST) influence the conformational homogeneity and hence the three-dimensional structure of in vivo folded target proteins (Smyth et al. 2003). To date, however, there are no data about the impact of small affinity tags, such as the $(His)_6$-tag, on the protein conformation and, especially, the disulfide pattern when generating cysteine-containing proteins by recombinant expression.

Reprint requests to: Sven Rothemund, Interdisciplinary Center for Clinical Research, Inselstrasse 22, D-04103 Leipzig, Germany, e-mail: sven_r@yahoo.com; fax: +49-341-97-15979; or Jana Klose, Research Institute of Molecular Pharmacology, Robert-Roessle-Str. 10, D-13125 Berlin, Germany; e-mail: jana_klose@yahoo.com; fax: +49-30-94793-159.

Abbreviations: ACN, acetonitrile; BSA, bovine serum albumin; CDAP, cyanodimethylaminopyridinium tetrafluoroborate; CRF, corticotropin-releasing factor; DTT, dithiothreitol; GPCR, G protein–coupled receptor; GST, glutathione S-transferase; GuHCl, guanidinium hydrochloride; HPLC, high-performance liquid chromatography; IAA, iodoacetamide; MALDI, matrix-assisted laser desorption/ionization; MBP, maltose binding protein; MS, mass spectrometry; NT, N terminus; RP, reversed-phase; SPA, scintillation proximity assay; TOF, time of flight.

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The large N-terminal domains of G protein–coupled receptor (GPCR) class B, such as the receptor for the peptide hormone corticotropin-releasing factor (CRF; Vale et al. 1981), contain several cysteines. There are two receptor subtypes for CRF in mammals, the CRF_1 and CRF_2 receptor (Chang et al. 1993; Chen et al. 1993; Sklenar et al. 1993; Vita et al. 1993; Lovenberg et al. 1995; Stenzel et al. 1995; Smith 2000), for the latter three splice variants, $CRF_{2(a)}$, $CRF_{2(b)}$ and $CRF_{2(c)}$, exist. The CRF_1 and $CRF_{2(b)}$ receptor N terminus (NT) without their putative signal sequence contain six cysteines (C1 through C6) and form three disulfide bridges. In contrast, the $CRF_{2(a)}$ and the $CRF_{2(c)}$ receptor NT without signal peptides exhibit only five cysteines (C2 through C6) and therefore forms only two disulfide bridges, leaving one cysteine unbound (Fig. 1). Recent studies on the N-terminal domains of the CRF receptors (CRF_1 receptor [Hofmann et al. 2001; Perrin et al. 2001], $CRF_{2(b)}$ receptor [Perrin et al. 2003]), as well as other receptors of this class, such as PTHR1 (Grauschopf et al. 2000) and GLPR1 (Bazarsuren et al. 2002), showed that the N-terminal domains serve as the major ligand binding site. For the CRF_1 receptor, it is also known that the disulfide pattern of the N-terminal domain is critical for binding of the natural ligand CRF (Qi et al. 1997). Thus, a defined conformation of the N-terminal domains of CRF receptors is suggested to be important for interactions with the ligands.

In this study, we investigated the disulfide patterns of recombinant, in vitro, folded N-terminal domains of the $CRF_{2(a)}$ receptor in dependence on the position of a $(His)_6$-tag. The binding behavior of several ligands (Table 1) to the refolded, C- or N-terminally, $(His)_6$-tagged receptor NT was studied by ligand binding assay by using the SPA technique.

Results and Discussion

The recombinant production of cysteine-containing proteins in *Escherichia coli* in inclusion bodies requires an in vitro folding step. The in vitro folding efficiency of disulfide bond–containing proteins is adjustable by several parameters, such as pH, protein concentration, ratio of low-molecular-weight thiols in reduced and oxidized form (Saxena and Wetlaufer 1970), and the concentration of non-denaturating reagents. The influence of other impacts, such as fusion tags on disulfide bridging, has to be elucidated for each individual protein.

Overexpression and in vitro folding

To investigate the influence of an N- and C-terminal $(His)_6$-tag, respectively, on the in vitro folding and resulting disulfide pattern of the five cysteine containing $CRF_{2(a)}$ receptor NT, both recombinant proteins were produced in *E. coli* (Fig. 1). According to different algorithms (Persson and Argos 1996; Cserzo et al. 1997; Nielsen et al. 1997; Reczko et al. 2002), amino acids Ala^{19}-Arg^{114} form the extracellular N-terminal domain of the rat $CRF_{2(a)}$ receptor without signal peptide. Overexpression of the $CRF_{2(a)}$ receptor NT with an N- and C-terminal $(His)_6$-tag, respectively, gave 40 to 60 mg *N*-$(His)_6$-$CRF_{2(a)}$ receptor NT and 30 to 60 mg $CRF_{2(a)}$-*C*-$(His)_6$ receptor NT per liter cell culture. Here, the position of the $(His)_6$-tag had no remarkable influence on the expression levels. Sodium dodecylsulfate–polyacrylamide gel electrophoresis of soluble and insoluble protein fractions showed that both $CRF_{2(a)}$ receptor NTs were deposited in inclusion bodies almost exclusively, and that the apparent molecular size of both $CRF_{2(a)}$ receptor NT is ~12.5 kDa according to the predicted molecular size. It should be noted that the presence of the $(His)_6$-tag on both termini led to increased expression levels in comparison to the untagged protein.

For subsequent protein renaturation, an in vitro refolding protocol according to procedures described elsewhere (Buchner and Rudolph 1991; Rudolph and Lilie 1996; Lilie et al. 1998) was applied. The optimized protocol led to a rapid reshuffling of improper disulfide bonds and to an enrichment of one folding species for the N- and C-terminally $(His)_6$-tagged protein, respectively, with yields of renaturation in a range of 40% to 50%. Because of the requirement of a high resolution for the separation of folding intermediates, preparative reversed-phase high-performance liquid chromatography (HPLC) was used to purify the oxidized products, with an overall yield of 10% for the entire folding and purification process.

Assignment of disulfide bridges

The characterization of disulfide patterns in the extracellular N-terminal domains of the N- and C-terminally $(His)_6$-

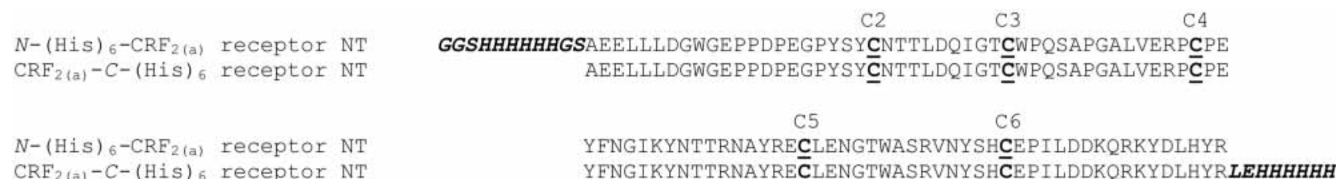

Figure 1. Alignment of the expressed $CRF_{2(a)}$ receptor N termini with an N- and C-terminal $(His)_6$-tag, respectively.

Table 1. *Primary structure of CRF analog*

Peptide	Sequence
Rat urocortin 1 (1–40)	D D P P L S I D L T F H L L R T L L E L A R T Q S Q R E R A E Q N R I I F D S V
Rat urocortin 1 (8–40)	- - - - - - - D L T F H L L R T L L E L A R T Q S Q R E R A E Q N R I I F D S V
Astressin[a]	f H L L R E V L E B A R A E Q L A Q E A H K N R K L B E I I * *

All peptides were synthesized as C-terminal amides. The truncated rat urocortin 1 (8–40) is N-terminally acetylated.
[a] f indicates D-phenylalanine; B, norleucine.
* Lactam bridge connecting the side chains of glutamic acid and lysine.

tagged $CRF_{2(a)}$ receptors was done by a combination of enzymatic/chemical cleavage and mass spectrometry (MS) of cleaved fragments. The homogeneity of the digested, final, purified refolding products was confirmed by the lack of differently connected species. It should be noted that $CRF_{2(a)}$ receptor NT carries only five cysteines (C2 through C6); consequently, only two disulfide bridges and one unlinked cysteine can be expected. Moreover, a chemical cleavage at Asn^{69}-Gly^{70} by hydroxylamine (Bornstei and Balian 1970) before the Glu-C digestion was necessary to increase the susceptibility of *N*-$(His)_6$-$CRF_{2(a)}$ receptor NT for the enzyme. The following analysis of digested fragments revealed the linkages Cys^{64}(C4)–Cys^{98}(C6) and Cys^{40}(C2)–Cys^{50}(C3) leaving Cys^{83}(C5) as unbound (Table 2). For the $CRF_{2(a)}$-*C*-$(His)_6$ receptor NT, we faced the problem of disulfide scrambling (Glocker et al. 1995) due to the uneven number of cysteines when performing standard digestion at pH 7.5. For this case, we used the strategy for blocking the free cysteine applying alkylation (Sechi and Chait 1998) or cyanylation (Wakselman et al. 1976) at pH 5.7 with iodoacetamide (IAA) and cyanodimethylaminopyridinium tetrafluoroborate (CDAP), respectively. After enzymatic digestion of the alkylated or cyanylated NT using chymotrypsin, the found fragments clearly show disulfide bridging between Cys^{40}(C2) and Cys^{83}(C5) as well as Cys^{64}(C4) and Cys^{83}(C6) (Table 2). We concluded from these unambiguously obtained disulfide bridges that Cys^{50}(C3) is not involved in disulfide bonds. Thus, the disulfide pattern of *N*-$(His)_6$-$CRF_{2(a)}$ receptor NT is not analog with disulfide bridges C1–C3, C2–C5, and C4–C6 found in both, CRF_1 receptor NT (Hofmann et al. 2001; Perrin et al. 2001) and $CRF_{2(b)}$ receptor NT (Perrin et al. 2003), respectively, which were expressed as soluble, in vivo, folded proteins. In opposite, the $CRF_{2(a)}$-*C*-$(His)_6$ receptor NT shows a disulfide pattern in accordance with the described ones for CRF_1 receptor NT and $CRF_{2(b)}$ receptor NT, respectively (Fig. 2).

The results unambiguously show that depending on the position of the $(His)_6$-tag, two dissimilar patterns were assigned for the two differently $(His)_6$-tagged $CRF_{2(a)}$ receptor NT (Fig. 2). This unexpected result can only be explained by an unfavorable influence of the $(His)_6$-tag on the folding behavior of the corresponding protein due to its close proximity to cysteine C2 and, second, a proposed less-stabilized conformation in this region of the $CRF_{2(a)}$ receptor NT. The latter aspect would consequently suggest a higher conformational stability in the region involving

Table 2. *Disulfide analysis of $CRF_{2(a)}$ receptor NT determined by MALDI-MS of cleaved fragments*

Fragment	Cysteine	Cysteine pattern	Calc. $[M+H]^+$	Found $[M+H]^+$
N-$(His)_6$-$CRF_{2(a)}$ receptor NT, hydroxylamine, GluC				
35-61	Cys40-Cys50	C2-C3	2869.30	2869.40
62-66+87-103	Cys64-Cys98	C4-C6	2546.20	2548.81
$CRF_{2(a)}$-*C*-$(His)_6$ receptor NT, chymotrypsin, cyanylation				
40-44+81-89	Cys40-Cys83	C2-C5	1655.71	1655.74
38-59+81-84[a]	Cys40,50-Cys83	C2,3-C5	2868.42	2868.21
45-68+96-102[a]	Cys50,64-Cys98	C3,4-C6	3486.93	3486.90
$CRF_{2(a)}$-*C*-$(His)_6$ receptor NT, chymotrypsin, alkylation				
39-43+80-88	Cys40-Cys83	C2-C5	1655.70	1655.50
59-67+95-108	C64-Cys98	C4-C6	2868.31	2868.30
39-58+80-88[b]	Cys40,50-Cys83	C2,3-C5	3486.80	3485.48

[a] Cyanylation adduct.
[b] Glutathione adduct.

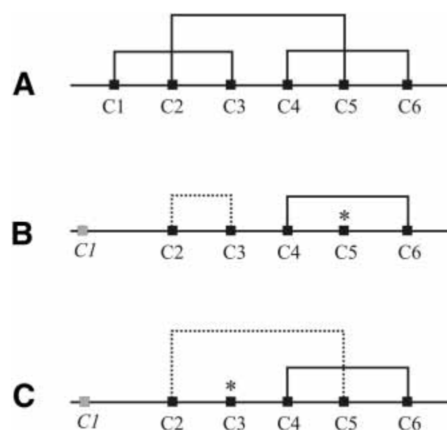

Figure 2. Disulfide pattern of the expressed, in vitro refolded $CRF_{2(a)}$ receptor N termini with an N- and C-terminal $(His)_6$-tag (*B*,*C*), respectively, in comparison to the in vivo folded CRF_1 receptor N terminus (*A*). Unbound cysteines are marked with *. Cysteine 1 in $CRF_{2(a)}$ receptor N termini is located in the signal sequence and labeled in italics.

cysteines C4 and C6, where independence of cysteine-linkage on the position of the $(His)_6$-tag was observed.

Ligand binding

The binding characteristics of selected ligands (Table 1) to the two N-terminal domains of the $CRF_{2(a)}$ receptor, exhibiting different disulfide patterns due to the position of the $(His)_6$-tag, were estimated by competitive displacement studies of radiolabeled rat urocortin 1 using SPA technique. Here, the anchorage of the extracellular N-terminal domains on copper-loaded SPA beads was mediated via the $(His)_6$-tag. The binding data (Table 3) show no significant differences in binding behavior of the two N-terminal domains of the $CRF_{2(a)}$ receptor (Fig. 3). Both domains show high affinities for the antagonist astressin (~5 nM) and moderate affinities for the N-terminally truncated rat urocortin 1(8–40; 50 to 70 nM), which in this assay is equipotent to the full-length rat urocortin 1(1–40). The completely reduced and ensuing alkylated $CRF_{2(a)}$ receptor NT (C-terminal $(His)_6$-tag) did not show any binding.

In summary, our results show that a "locking in place" of disulfide bonds in the course of oxidative in vitro folding of proteins can be influenced by the position of the $(His)_6$-tag. The isolated N-terminal domain of the $CRF_{2(a)}$ receptor containing an uneven number of cysteines exhibits two dissimilar disulfide patterns depending on the tag position, with varied C2/C3/C5 bonding but a constant C4–C6 disulfide bond. On the other hand, as shown for the $CRF_{2(a)}$ receptor NT, the occurrence of different disulfide patterns does not necessarily result in an altered binding behavior. Obviously, the conformation of the N-terminal portion of extracellular N-terminal domains of CRF receptors, especially the correctness of the disulfide bonds in this region, is not essential for ligand binding. In accordance with this finding are mutation studies of the CRF_1 receptor (Qi et al. 1997): Replacement of the first cysteine (C1) by serine did not affect the function. However, a completely reduced and ensuing alkylated $CRF_{2(a)}$ receptor NT [C-terminal $(His)_6$-tag] did not show any binding anymore, indicating the importance of the disulfide bridge between C4 and C6 and hence stability of this C-terminal region within the extracellular N-terminal receptor domain.

Table 3. *Results of ligand binding studies by SPA*

	$CRF_{2(a)}$-*C*-$(His)_6$ receptor NT	*N*-$(His)_6$-$CRF_{2(a)}$ receptor NT
Ligand	EC_{50} [nM]	EC_{50} [nM]
Rat urocortin 1 (8–40)	45.6 ± 4.9	71.0 ± 10.1
Astressin	6.8 ± 1.5	4.6 ± 0.5

The data are means of at least three different experiments performed in triplicates using $[^{125}I]Tyr^0$-urocortin as radioligand.

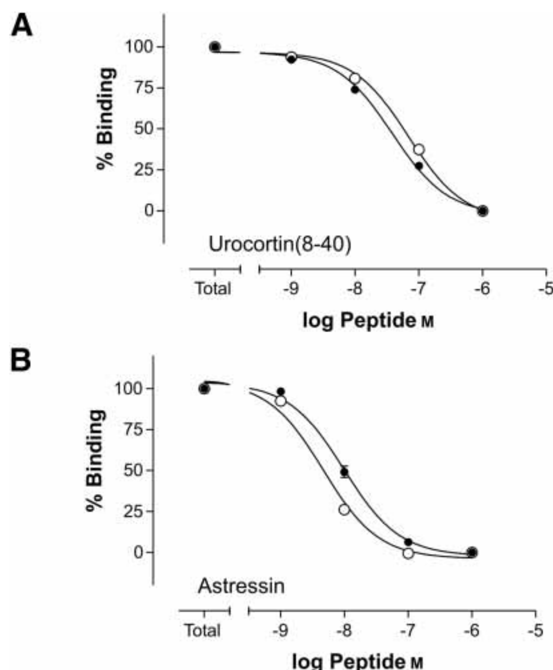

Figure 3. Inhibition of specific $[^{125}I\text{-}Tyr^0]$-urocortin 1 binding to CRF-rNT by the unlabeled CRF-like ligands urocortin 1 (8–40) and astressin by using the SPA technology. All values are given as mean ± SEM. (*A*) Binding of urocortin 1 (8–40) to the $CRF_{2(a)}$ receptor N terminus with C-terminal $(His)_6$-tag (filled circles) and N-terminal $(His)_6$-tag (open circles). (*B*) Binding of astressin to the $CRF_{2(a)}$ receptor N terminus with C-terminal $(His)_6$-tag (filled circles) and N-terminal $(His)_6$-tag (open circles).

Materials and methods

Construction of expression plasmids

N-$(His)_6$-$CRF_{2(a)}$ receptor NT

A PCR-amplified cDNA (312 bp, primers 5′-AGCTTGGGATC CGCCGAAGAGCTGCTTTTG-3′ and 5′-GATGAGCTCTTAT CGGTAATGCAGGTC-3′) coding for Ala^{19}-Arg^{114} of the rat $CRF_{2(a)}$ receptor was initially inserted into pQE-30 (Qiagen) via BamHI and SacI restriction sites. For expression reasons, the resulting vector was used as template for recloning a cDNA (348 bp) coding for rat $CRF_{2(a)}$ receptor NT, including an N-terminal $(His)_6$-tag (primers 5′-TTAACCATGGGAGGATCGCATCAC CAT-3′ and 5′-CGGGGTCTCGAGCTCTTATCGGTAATG-3′) into pET-15b (Novagen), using NcoI and XhoI restriction sites to yield the N-terminally $(His)_6$-tagged protein.

$CRF_{2(a)}$-C-$(His)_6$ receptor NT

To yield the C-terminally $(His)_6$-tagged protein, a cDNA (310 bp, primers 5′-CAGCTTGCATATGGCCGAAGAGCTGCTTTTGG-3′ and 5′-GACCTCGAGTCGGTAATGCAGGTCATACTTCC-3′) coding for Ala^{19}-Arg^{114} of the rat $CRF_{2(a)}$ receptor was inserted into pET-21a (Novagen) by using NdeI and XhoI restriction sites.

The authenticity of the resulting recombinant expression vectors was confirmed by DNA sequence analysis.

Expression in E. coli *and isolation of inclusion bodies*

E. coli BL21(DE3) (Novagen) was transformed with the respective plasmids and grown in LB medium supplemented with ampicillin (100 μg/mL). For expression, 1 L LB medium was inoculated with 15 to 50 mL overnight culture and grown at 37°C to an optical density of OD_{600nm} = 0.5 to 0.7. Expression of the proteins was induced with 1 mM IPTG for 3.0 to 3.5 h at 37°C. Cells were harvested by centrifugation, and the cell pellets were resuspended in 20 mM Tris, 2% (v/v) Triton, and protease inhibitor cocktail (EDTA-free at pH 8; 15 mL/pellet from 1 L cell culture). The cells were disrupted by high-pressure dispersion and subjected to lysis, and the inclusion bodies were collected and stored at −20°C.

The sequences of the expressed proteins are shown in Figure 1.

Renaturation and purification

The inclusion body pellet was solubilized in denaturation buffer (5 M guanidinium hydrochloride [GuHCl], 20 mM Tris at pH 7.5) by shaking and sonication. The proteins were purified by immobilized-metal affinity chromatography by using a chelating Sepharose FF column (Amersham Pharmacia Biotech AB) with immobilized Ni^{2+} ions (loading and washing buffer: 5 M GuHCl, 20 mM Tris at pH 7.5, elution buffer: 5 M GuHCl, 20 mM Tris, 0.5 M imidazole at pH 7.5). Following purification, the proteins were reduced by addition of dithiothreitol (DTT; 100 mM, 1 to 2 mg protein/mL, room temperature for 2 h). After reduction of the proteins, DTT and Ni^{2+} ions were removed by dialysis against denaturation buffer (pH 3.0) at 10°C. After readjusting the pH to 7.5, renaturation was achieved by dialysis against 0.5 M L-arginine, 100 mM Tris, 1 mM EDTA, 1 mM reduced glutathione (GSH), 1 mM oxidized glutathione (pH 7.5), and 0.5 to 0.7 mg protein/mL for 3 d at 10°C. After renaturation, a final dialysis step against 10% (v/v) glycerol, 50 mM NaCl, and 20 mM Tris (pH 6.5) overnight at 10°C was accomplished, and insoluble material was removed by centrifugation. The refolded proteins were purified by reversed phase (RP)–HPLC by using a Vydac C4 column (10 × 250 mm, 5-μm particle size, 300 Å pore size, number 214TP510), run in 0.1% (v/v) trifluoroacetic acid in water with increasing concentrations of acetonitrile (ACN) as mobile phase, and lyophilized.

Peptides

Astressin was purchased from Bachem AG. [^{125}I-Tyr^{0}]-urocortin 1 (2200 Ci/mmole) was obtained from Amersham Biosciences Europe GmbH. Other peptides were synthesized following the procedure described in reference (Beyermann et al. 2000).

Disulfide pattern analysis

Disulfide pattern analysis of all CRF receptor NTs was carried out by standard procedures of enzymatic digestion using trypsin, chymotrypsin, or Glu-C followed by matrix-assisted laser desorption/ionization (MALDI)–MS.

N-(His)$_6$-CRF$_{2(a)}$ receptor NT

Before analyzing the disulfide pattern in *N*-(His)$_6$-CRF$_{2(a)}$ receptor NT, the protein was treated with 1.8 M hydroxylamine (Bornstei and Balian 1970) and incubated for 5 h at 45°C for cleavage of the Asn^{69}-Gly^{70} and the Asn^{86}-Gly^{87} peptide bonds. Following cleavage, the protein was purified by RP-HPLC (see Renaturation and Purification).

CRF$_{2(a)}$-C-(His)$_6$ receptor NT

For analyzing the disulfide pattern of CRF$_{2(a)}$-*C*-(His)$_6$ receptor NT, free cysteines were blocked by alkylation with IAA (phosphate buffer at pH 5.7, 25 μM protein, 5 mg/mL IAA, 2 M GuHCl, overnight at 37°C) or cyanylation with CDAP (phosphate buffer at pH 5.7, 25 μM protein, 0.25 mM CDAP, 1 mM EDTA, overnight at 40°C). Removal of excess reactants was performed by dialysis against 2 M GuHCl, 20 mM Tris, and 1 mM EDTA (pH 7.5).

MALDI-MS measurements were performed on a Voyager-DE STR BioSpectrometry Workstation MALDI–time of flight (TOF) mass spectrometer (Perseptive Biosystems, Inc.). As matrices for analyses of peptides and proteins, α-cyano-4-hydroxycinnamic acid and sinapinic acid, respectively, were used. The program SearchXLinks (www.caesar.de/searchxlinks/) was used to analyze the mass spectra of protein digests with regard to the presence of disulfide-linked fragments.

Ligand binding assay: SPA

The competitive binding assays were performed in triplicates in 2.0-mL colorless reaction tubes (Biozym Diagnostik GmbH) at room temperature by using a PVT copper His-tag SPA bead suspension (Amersham Biosciences Europe GmbH) in assay buffer containing 0.1% bovine serum albumin (BSA). The following reagents, diluted in assay buffer, were added in the order: 100 μL of unlabeled peptide with increasing peptide concentrations or buffer, 75 μL [^{125}I-Tyr^{0}]-urocortin (120 pM final concentration), and 75 μL of the respective CRF$_{2(a)}$ receptor NT (20 ng/tube). After incubation of the reaction mixture for 2 h, 50 μL of 10 mg/mL bead suspension (500 μg beads/tube) were added. The final reaction mixture was shaken and then incubated for 4 h. Finally, the tubes were counted in a Wallac 1410 set up in a ^{3}H cpm-mode (SPA-cpm). Total binding observed and normalized at 100 pM; final concentration of tracer was ~13,000 SPA-cpm for both CRF$_{2(a)}$ receptor NT, with a nonspecific signal of ~3000 SPA-cpm determined in the presence of unlabeled 1 μM astressin. Binding data were analyzed by using the GraphPad Prism software.

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