Published in final edited form as:

Biochemistry. 2008 December 16; 47(50): 13308–13317.

The Solution Structure of a Conformationally Restricted Fully Active Derivative of the Human Relaxin-like Factor (RLF)[†]

Erika E. Büllsbach^{‡, *}, Mathias A.S. Hass^{§, †}, Malene R. Jensen^{§, ‖}, D. Flemming Hansen^{§, ¶}, Søren M. Kristensen[§], Christian Schwabe[‡], and Jens J. Led. [§]

[‡]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Avenue, P.O. Box 250509, Charleston, South Carolina 29425, U.S.A

[§]Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen Ø, Denmark

Abstract

Analogous to insulin, the relaxin-like factor (RLF) must undergo a structural transition to the active form prior to receptor binding. Thus, the C terminus of the B chain of RLF folds toward the surface of the central B chain helix causing partial obliteration of the two essential RLF receptor-binding site residues valine-B19 and tryptophan-B27. Comparing the solution structure of a fully active C-terminally cross-linked RLF analog with native synthetic human RLF (hRLF) it became clear that the cross-linked analog largely retains the essential folding of the native protein. Both proteins exist in a major and minor conformation, as revealed by multiple resonances from tryptophan-B27 and adjacent residues on the B chain helix. Notably the minor conformation is significantly higher populated in the chemically cross-linked RLF than it is in hRLF. In addition, compared to the unmodified molecule subtle differences are observed within the B chain helix whereby the cross-linked derivative shows reduced hydrogen-bonding and significant peak broadening at the binding-site residue ValB19. Based on these observations we suggest that the solution structure of the native hormone represents an inactive conformer and that a dynamic equilibrium exists between the C-terminally unfolded binding conformation and the inactive conformation of RLF.

Keywords

NMR; ¹H; ¹⁵N; protein; relaxin-like factor; structure; function; INSL3; receptor-binding conformation; cross-linked RLF

The relaxin-insulin family consists of eight structurally related hormones and growth factors: insulin (1), insulin-like growth factors (IGF) (2), relaxin (3,4), relaxin-like factor (RLF) also

[†]This work was supported by Grants GM 48893 and 1-R01-HD40406 from the National Institutes of Health, by the Medical University of South Carolina, Research Committee, the Danish Agency for Science, Technology and Innovation Grants 9400351, 9801801, 26-03-0055, 21-04-0519 and 272-07-0466, Carlsbergfondet Grant 1624/40, Novo Nordisk Fonden Grant 2003-11-28, and Villum Kann Rasmussen Fonden Grant 8.12.2003.

*Corresponding author Editorial correspondence should be sent to: Erika E. Büllsbach, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 173, Ashley Avenue, P.O. Box 250509, Charleston, South Carolina 29425, Telephone: 843-792-9926, Fax: 843-792-4850, E-mail: bullesee@musc.edu.

[‡]Present address: Leiden Institute of Chemistry, Leiden University, Gorlaeus Laboratories, P.O. Box 9502, 2300 RA Leiden, The Netherlands

[‖]Present address: Institut de Biologie Structurale Jean-Pierre Ebel, 41 Rue Jules Horowitz, 38027 Grenoble, France

[¶]Present address: Department of Medical Genetics and Microbiology, University of Toronto, 1 King's College Circle, Toronto, ON, M5S 1A8 (Canada)

^{††}Structures are deposited in the protein data bank with the entries 2k6t for human RLF and 2k6u for cross-linked RLF derivative.

named insl3, or Ley-IL) (5), placentin (or insl4) (6), insl5 (7), insl6 (8) and insl7 (also named relaxin 3) (9,10). With the exception of the insulin-like growth factors (IGF) all proteins consist of two polypeptide chains, the A chain and B chain, held together by three disulfide bonds in conserved spacing. Two disulfide links connect the A and B chain while the third disulfide bond appears within the A chain. Three-dimensional structures of insulin (11), IGF (12,13), relaxin (14,15) and the relaxin-like factor (RLF) (16) indicate that the intra-A chain disulfide bond stabilizes the A chain-loop and the two antiparallel short helical segments located in the N and C-terminal region of the A chain. In all structures a longer helix is found in the mid-region of the B chain, which often is followed by a β -turn. Functionally the hormones are quite diverse, for instance, insulin and IGF exhibit their metabolic / growth promoter function by acting through tyrosine-kinase receptors (17-19), whereas relaxin and RLF stimulate tissue remodeling through 7-transmembrane G-protein coupled receptors (10,20-22).

RLF is a critical regulator of sexual differentiation that mediates intra-abdominal migration of the testes in the developing male (23). The protein isolated from bovine testis consists of a 26 amino acid residue A chain and a 41-residue B chain and is significantly larger than insulin or relaxin. In its native form the binding affinity to the receptor appears reduced when compared to a shorter form deduced from the cDNA sequence (24) in which the B chain consists of the N-terminal 31 residues. While this form is almost exclusively used in structure and function studies it has been shown that the C-terminus of the RLF B chain can even be further shortened to 27 residues, ending in TrpB27 amide (25).

RLF binds the leucine-rich G-protein coupled receptor-8 (LGR8), with remarkable affinity ($K_d = 0.1$ nM) (26) despite lack of a sharply defined binding site (16,27,28). Single residue replacements (16,25,28,29) revealed that the side chains of ArgB16, ValB19 and TrpB27 are important but made it clear that the sum of individual side chain contributions of RLF does not account for the observed high affinity binding constant. In contrast, multiple replacements of these same residues, or of residues adjacent to the binding residues caused an effect that was significantly larger than the sum of the effects of single replacements. This implies that the proper orientation of other, secondary binding sites is necessary for receptor-binding (16,28). Such a mechanism of RLF-receptor interaction should allow one to “freeze” the molecule in a purported active conformation. In a previous paper we reported on the synthesis and properties of a series of derivatives containing cross-links of variable length between the side chain in position B26 and the C-terminus of the A chain. The results of that study led to the conclusion that the distance between the two chains is critical for hormonal activity. Accordingly, cross-linked RLF bearing either a glycine or a β -alanine spacer are fully active, while direct linking of the A chain C terminus and B26 lysine led to a loss of 97% of the receptor-binding capacity (25). In addition, it was found that the average position of side chains varied considerably in the differently cross-linked RLFs, and that the restriction of motion of TrpB27 by cross-linkers of various lengths influences the activity of the hormone. These observations pointed to the exciting possibility that the different cross-links freeze RLF at various distances from its binding conformation. We have determined the solution structure of a C-terminally cross-linked RLF analog that is fully active. As suggested by our analysis of the structure of the analog as well as the native RLF both proteins must undergo a structural transition to the active form prior to receptor binding.

Materials and Methods

Materials

Human RLF (hRLF) and ^{15}N (GlyB23, GlyB24, AlaA2, AlaA7, GlyA14)-labeled hRLF were synthesized as previously described (29). Cross-linked human RLF consisted of a 26-residue A chain extended C-terminally by glycine-A27, a 27-residue B chain with arginine-B26 replaced by lysine and with an amidated C terminus. The ϵ -amino group of lysine-B26 and the

carboxyl group of glycine-A27 were condensed to establish an isopeptide cross-link between the A and B chain. The synthesis has been published (25).

NMR spectroscopy

The protein was dissolved in 40 mM perdeuterated acetic acid/NaOD, pH 5.0, in water containing 8% D₂O or in D₂O. NMR tubes were sealed under nitrogen shortly after sample preparation. NMR experiments were conducted at a magnetic field strength of 18.7 T using a Varian Unity Inova 800 spectrometer equipped with a cryo-probe. The water signal was suppressed either by selective excitation of the water resonance (WATERGATE) (30) or by pre-saturation of the residual HDO resonance of samples in D₂O. In all experiments the carrier frequency was set at the center of the spectrum at the water resonance. Three different types of two-dimensional ¹H-¹H correlated NMR experiments were conducted: DQF-COSY (31), NOESY (32,33) and TOCSY (34,35). ¹H-¹³C HSQC spectra of the aliphatic region were acquired at a magnetic field strength of 18.7 T. The sweep width was 12 kHz in the ¹H dimension and 16 kHz in the ¹³C dimension. Spectra were processed using the NMRPipe software package (36) while spectral analyses were carried out with the program SPARKY (by T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco).

For hRLF TOCSY and NOESY conditions were optimized by recording spectra with different mixing times (40 ms, 60 ms, 80 ms, 100 ms for TOCSY; 80 ms, 120 ms, 200 ms for NOESY) and temperatures (20°C, 23°C, 28°C, 30°C, 33°C, 35°C, 40°C). The optimized conditions (mixing times of 60 ms for TOCSY and 120 ms for NOESY) and temperatures of 25°C, 30°C, 35°C were applied for cross-linked RLF. Severe ambiguities in peak assignments were resolved by evaluating spectra at different temperatures. To investigate inter-molecular interactions a series of NOESY spectra of hRLF were recorded at 35°C and pH 5.0 at concentrations of 1.2 mM, 0.6 mM, 0.3 mM, 0.2 mM, and 0.1 mM. The number of scans per FID was increased throughout the concentration series from 16 scans at the highest concentrations to 64 scans at the lowest.

Structure calculations

Sequential assignment was accomplished using the strategy described by Wüthrich (37). Most spin systems were identified but AlaA7 was only assigned after ¹⁵N-labeled amino acids were incorporated into hRLF positions GlyB23, GlyB24, AlaA2, AlaA7, and GlyA14. ¹H-¹⁵N HSQC and ¹H-¹⁵N HSQC-NOESY experiments were conducted to identify proton resonances of the amide and methyl groups. For structure calculations interproton distance restraints were obtained from NOESY spectra at 35°C and a mixing time of 120 ms. Deuterium exchange experiments at pH 5.0 and 35°C failed due to fast exchange of all amide protons indicating the presence of a significant population of partially unfolded structures besides the folded form. However, at pH 2.7 and 15°C slow exchanging protons were identified and were essentially identical to backbone amides of helical segments as predicted by the chemical shift index (38) and by the amide proton temperature coefficients (39). Thus, the α -helices B12-B21, A5-A12, and A17-A22 were predicted and thirteen H-bond restraints were included in the calculations.

^H^N-H^A coupling constants for hRLF were determined by a combination of linear predictions and least square analyses (40). The ϕ angles in predicted helical regions could not be resolved by this procedure but were predicted using the program PREDITOR (41). For hRLF 20 coupling constants and 22 predicted ϕ and 19 predicted ψ torsion angles were included in the final calculations, whereas for cross-linked RLF 25 predicted ϕ and 18 predicted ψ bond angles were used. Based upon intra-residue NOE intensities (42) 12 and 8 χ torsion angles were included for hRLF and cross-linked RLF, respectively.

The software ARIA (Ambiguous Restraints for Interactive Assignment, versions 2.0 or 2.1) (43) in combination with CNS (44) was used for structure calculations. The input consisted of two NOESY spectra acquired in H₂O and in D₂O, respectively, at pH 5.0, at 35°C and a mixing time of 120 ms. The data set recorded in H₂O included all resonances involving aromatic side chains and amide protons, whereas the data set recorded in D₂O included only resonances between aliphatic protons. Peaks were selected on one side of the diagonal only to ensure unique entry. In addition, all spectra were carefully screened and artifacts were excluded. This measure reduced the number of both merging and rejected NOEs during the structure calculation and eased the evaluation of those peaks. Unambiguous long-range and medium-range NOEs were pre-assigned.

For iterations 0 to 8 in the ARIA calculations, the violation tolerance was set at 1000 Å, 5 Å, 3 Å, 1 Å, 1 Å, 1 Å, 0.5 Å, 0.3 Å, and 0.3 Å, respectively. The frequency windows were set to 0.026 ppm and 0.013 ppm in the F₁ and F₂ dimension, respectively. This setting eliminated the frequency of merging unrelated peaks and reduced the NOEs rejected by the program (45) (30 for hRLF and 6 for cross-linked RLF).

Results

The structures of human RLF and the fully active C-terminally cross-linked RLF derivative were obtained from the experimental NMR data and compared. The cross-link was constructed by extending the C-terminus of the A chain by glycine-A27 and by replacing arginine-B26 in the B chain by lysine. Condensation of the carboxyl group of GlyA27 to the side chain amino group of LysB26 established a permanent cross-link within the C-terminal region of the molecule. The sequences of hRLF and the cross-linked analogue are displayed in figure 1.

Sample conditions and spectral parameters were optimized for human RLF and subsequently applied to the cross-linked derivative. CD-spectroscopy in the far-UV region indicated that secondary structure changes occur below pH 3.0 (Fig. S2, supporting information). On the other hand, at neutral pH the solubility in water was reduced to approximately 0.1 mM. We have chosen pH 5.0 because at this pH hRLF retains a high solubility (>1.2 mM) and because amide proton exchange is slower than it is at physiological pH while the native conformation is maintained as judged by the far-UV CD measurements.

Backbone chemical shifts within a concentration series for hRLF showed that concentration-dependent differences were small, with the largest shifts observed (0.02 - 0.04 ppm) for amide protons ValB15, ValB19, ArgB20, ValB21 and the α -protons of GlyB24 (Fig. 2). The small changes in chemical shift indicate a weak self-association of RLF. This was confirmed by hydrodynamic measurements based on ¹⁵N NMR relaxation (Fig. S3, supporting information). Close inspection of the NOESY spectra did not reveal any specific concentration-dependent cross-peaks that would be indicative of inter-molecular interactions. The structure of both proteins was predicted based on the NOESY spectra that were recorded at a protein concentration of 1.2 mM. The structures were calculated using the software ARIA (Ambiguous Restraints for Interactive Assignment), an interactive protocol for simultaneous NOE-assignment and structure calculation (43).

Structure of human RLF

The chemical shifts of the amide protons were well dispersed ranging from 9.7 ppm for ThrA16 to 7.0 ppm for PheB14/ValB15. Sequential, short and medium range NOEs (Fig. 1A) provide clear evidence for the presence of α -helices in the C-terminal region of the A chain (residues GlnA17 - ThrA22) and the mid-region of the B chain (residues HisB12 - ValB21). With the α -protons and α -carbons of AlaA7 and CysA10 unassigned, the presence of the helix in the N-terminal region of the A chain (ProA6 - LeuA12) was established by the slow hydrogen

exchange rates for the NH-protons of residues ArgA8, TyrA9, and LeuA12, by chemical shift-based calculations of the temperature coefficients for backbone amide protons (39), and by torsion angle predictions (41). Long-range NOEs between residues LeuB9, PheB14, AlaB17, and ValB21 of the B chain and ProA6, AlaA7, CysA10, CysA15, LeuA20 and LeuA23 of the A chain are indicative of a hydrophobic core (Fig. 3A and 3B). The relative orientation of the two antiparallel A chain helices is deduced from NOEs between the side chains of ProA6 and LeuA23 and by additional long-range NOEs between the phenol ring of TyrA9 and side chains of residues located in the N-terminal region of the A chain (residue AlaA3, ThrA4), the mid-region of the A chain (residues CysA15, ThrA16) and in the C-terminal region of the A chain (residue LeuA23). Numerous NOEs were observed between the N-terminal segment of the B chain (Arg-Glu-Lys, B6-B8) and the mid-region of the A chain (Gly-Cys-Thr, A14-A16). As reported by Rosengren *et al.* (16) two different signals were observed for the N-terminus of the B chain (B1-B6) probably representing trans- and cis-isomers of the Thr(B2)-Pro(B3)-bond. The C-terminus of the B chain folds back to the mid-region of the B chain, as signified by NOEs between the indole-ring protons of TrpB27 and the methyl-groups on the surface of the B chain helix (ValB15, LeuB18 and ValB19). The overall structure is similar to that reported by Rosengren *et al.* (16). However, the indole side chain of TrpB27 in our structure is differently oriented and more restricted in its position. Thus, long-range NOEs were observed between all aromatic protons of the indole ring and the methyl groups of ValB19, between all protons of the phenyl ring and the methyl groups of ValB15, and between protons H2 and Z2 of the indole ring and the methyl groups of LeuB18. Together these NOEs indicate that the active site residue TrpB27 is closer to the other active site residue, ValB19, and to residues ValB15 and LeuB18, than reported previously (16).

Structure of cross-linked RLF

The overall spectral features of the C-terminally cross-linked RLF are similar to those of native RLF. This includes the dispersion of the backbone amide resonances and the observation of multiple resonances for residues B1-B6. However, resonances display increased line-broadening reducing the total number of evaluated NOEs compared to hRLF (Table 1). At the N-terminal region of the A chain (Fig. 1B) only few medium-range NOEs indicative of an α -helix could be identified. Medium- and long-range NOEs (Fig. 3C) persisting between the side chain of TyrA9 and AlaA3, GlyA14, CysA15, ThrA16, AspA19 and LeuA23 are indicative of a folded structure. In addition, predictions based on chemical shift data (38,39,41) suggest the presence of an α -helical segment for the sequence ProA6 to LeuA12, as well as for the segments GlnA17 to ThrA22 and HisB12 to ValB21 in the cross-linked derivative. Long-range intra-A chain NOEs are more frequent in the cross-linked RLF than in hRLF, in particular NOEs between Pro(A6) and Leu(A23) as well as Tyr(A9) and Leu(A23). These additional NOEs are of no apparent consequence for the overall fold of the A chain. Fewer long-range NOEs were recorded for the mid- to C-terminal region of the B chain of cross-linked RLF as compared to hRLF (Fig. 3). The cross-link (LysB26, GlyA27) itself gave rise to intra-residue, sequential and short-range NOEs indicating that the link does not interfere with the overall fold of the protein and that it does not contribute to the structure other than restricting the distance between the C-terminal ends of the two chains.

Comparison of human RLF and its cross-linked derivative

Comparison of the chemical shift values of the spectra of the two proteins shows only small differences (Fig. 4). As expected, the largest deviations are observed around the position of the restricting cross-link within the C-terminal region of both chains (GlyB23 - TrpB27, and LeuA23 - TyrA26). Interestingly, the effect propagates mostly along the surface of the B chain helix affecting ($\Delta > |0.05|$ ppm) the amide protons of CysB10, GlyB11, HisB12, ValB15, LeuB18, ValB19, and ArgB20 and the alpha hydrogen of HisB12. The largest difference exceeding 0.1 ppm was recorded for the amide protons ValB15 and LeuB18. In contrast,

hydrophobic core residues PheB14, AlaB17 and ValB21 are less or not affected by the modification. Within the A chain, the differences were most pronounced ($\Delta > |0.05|$ ppm) for the cysteine amide protons A10, A11 and A15 and for the alpha proton of LeuA23. These small chemical shift differences do not reflect any difference in the secondary structure as predicted by the chemical shift index (Fig. 5).

Temperature coefficients less negative than -4.25 ppb/K are indicative of hydrogen bonded amide protons (39). Accordingly, hydrogen bonds were predicted for amide protons PheB14 through ValB21 suggesting the presence of the B chain helix (HisB12 to ValB21) as shown in Fig.6. Both helices of the A chain are predictable in a similar way, although with less certainty. The positive values for the temperature coefficients for PheB14, CysA11, and GlyA14 suggest that these amide protons are located in a hydrophobic environment (39). It is noteworthy that all but the temperature coefficient for TyrA26 are more negative for cross-linked RLF than for hRLF, indicating weaker hydrogen bonds and hence reduced stability and increased flexibility of the structure of the derivative. In fact, for cross-linked RLF, temperature coefficients for ValB15, AspA19, LeuA20 and LeuA21 are below the threshold whereas the temperature coefficients for ArgB20 and ValB21 are at the threshold for α -helix-stabilizing hydrogen bonds (Fig. 6).

From the NOEs it can be concluded that the aromatic side chains of TrpB27 in the C-terminal region of the native and the cross-linked protein have different orientations with respect to the B chain helix. Thus, in the cross-linked derivative only NOEs between the indole protons HD1 and HE3 and the ValB19 methyl groups are retained, whereas new NOEs appear between the phenyl ring protons E3 and Z3 and the methyl groups of LeuB18. In addition one methyl group of LeuB18 is upfield shifted by 0.12 ppm. Consequently, the TrpB27 indole-ring in cross-linked RLF occupies a position different from that in native RLF, which affects mostly the location of the phenyl moiety while the pyrrole rings of the two proteins are in similar positions relative to the rest of the protein (Fig. 7). This result is in agreement with the previously reported quenching of the tryptophan fluorescence in this cross-linked analogue (25). With the altered position of the indole ring in cross-linked RLF the methyl groups of ValB19 are now more solvent exposed.

Both hRLF and cross-linked RLF show multiple resonances for a series of residues on the B chain suggesting at least two conformations. The residues showing multiple resonances are TrpB27 and the residues ValB15, ValB19, and ValB21 on the B chain helix. For TrpB27 the relative intensities of the resonances corresponding to the two conformations are similar for both proteins (Fig.8). In addition, the relative intensities of corresponding resonance in the two conformers are different for the three valine-residues on the B chain helix that show double signals. Thus, for each of the valines at least two different spin-systems coexist in which chemical shifts are markedly different for the alpha and gamma protons. For instance, the intensities of the main HA-HG resonances for ValB15 in hRLF and cross-linked RLF are 90% and 73%, respectively, whereas for ValB21 the main resonance constitutes 98% in hRLF and is slightly asymmetrical, while it constitutes only 76% in the cross-linked derivative and is symmetrical. In case of ValB19 the HA-HG resonances in the TOCSY spectrum show two distinct double signals in a 1:5 ratio for hRLF, but in the cross-linked derivative the main two resonances have collapsed to a diffuse peak (Fig. 9). No sequential NOEs could be established between the signals corresponding to the minor forms and the different conformers were identified only by their different signals in the TOCSY spectra (Fig. 8, 9) and by the multiplicity of the ^1H - ^{13}C resonances in the ^1H - ^{13}C HSQC spectra. The subsequent structures were calculated using the main resonances (Fig. 10). However, the fact that multiple signals are observed shows that both RLF and cross-linked RLF assume two different conformations around TrpB27, corresponding to a major and a minor conformation. Moreover, we find that the population of the minor conformation is higher in the cross-linked RLF than in hRLF. On

the basis of these observations and the fact that the cross-linked RLF investigated here is the analog with the highest activity among those examined previously (25), we suggest that the minor conformation corresponds to the conformation that RLF must assume to bind to its receptor, as discussed in detail below.

Discussion

Alanine scanning of RLF has led to the identification of the side chains of ArgB16, ValB19 and TrpB27 as receptor binding residues (16,28). B chain truncation, tryptophan substitution and steric displacement experiments have shown that tryptophan-B27 is essential for binding (29). Thus, deleting the C-terminal region of the B chain, including TrpB27, reduced the affinity for the receptor 250-fold, and alanine substitution for tryptophan showed a 100-fold reduction in affinity (29). When TrpB27 was replaced by phenylalanine, or by D-tryptophan, or was oxidized to the oxindole the affinity was reduced 2- to 5-fold indicating that an aromatic interaction is essential for the receptor binding. This conclusion is supported by the fact that relaxin will cross-react with the RLF receptor only when a tryptophan is present in a position equivalent to TrpB27. Thus, human and porcine relaxins, with Trp in position B27 bind and activate the RLF receptor whereas rat relaxin with a glycine in position B27 does not (46).

However, despite the fact that the indole ring is necessary for high affinity binding, the solution structures for both hRLF and cross-linked RLF show that the binding residue, tryptophan-B27 and, in case of hRLF also valine-B19, are partially buried within a common fold and therefore not readily accessible. Consequently, the dominant solution structures of hRLF and the cross-linked derivative must represent non-binding conformers. The binding conformer may be best represented by the minor conformation observed for cross-linked RLF. Decreased stability of the B chain helix, significant line-broadening of the ValB19 side chain including improved surface exposure of the methyl groups, and two conformations of the indole-ring of TrpB27 all imply that a more open structure does exist in addition to the compact fold.

To convert the inactive form (T-state) to an active conformation (R-state) a partial unfolding must occur prior to receptor binding. Most likely, the T and the R states exist in an equilibrium from which the receptor recruits the binding conformer. Therefore, in spite of the different orientation of the tryptophan side chain in the T-state of hRLF and cross-linked RLF (Fig. 7), in the R-state of the two proteins the indole ring could occupy a very similar position in space. It was suggested that the GGP sequence (B23-B25) functions as a hinge in the orientation of the indole ring (28,29). Indeed, when L-ProB25 was replaced by its D-enantiomer or by L-serine the corresponding RLF derivatives showed substantially reduced receptor affinity (28, 29). In this context it is interesting that within the relaxin/insulin family, insulin also requires a transition from an inactive state prior to insulin-receptor binding. In this transition the flexibility of the C-terminal region of the insulin B chain is crucial to expose the binding site. X-ray (47) and NMR data (48,49) of native insulin show that the β -turn (B20-23) C-terminal of the B-chain helix directs residues B24-28 to the surface of the B chain helix, forming the dimerization site of the insulin hexameric storage form. This fold is maintained in the monomer when dissociating solvents are present (48,50). Molecular dynamic calculations implied that in the virtual insulin monomers the C-terminal portion of the B chain B25-B30 (equivalent to RLF-residues B28-B33) moves away from the B-chain helix to expose the receptor interacting site of the hormone (51). Such a movement is supported by structures of non-aggregating insulin mutants with unusually high receptor affinities (52-57). In insulin the β -turn (B20-23, GERG) is stabilized by two characteristic hydrogen bonds, one between the carbonyl oxygen of Gly(B20) and the NH-proton of Gly(B23) and the second between Cys(B19) carbonyl oxygen and Arg(B22) NH proton. In several flexible potent insulin analogs these H-bonds seem to be absent (54) or altered (56). Insulins mutated within the B20-23 β -turn suggest that neither the β -turn geometry nor its local stability correlate with activity implying that the highly

conserved β -turn is not essential for receptor affinity, in fact may represent inactive conformers (58).

In RLF the turn C-terminal of the B-chain helix (B23-26, GGPR) is not a type-I β -turn as in insulin because one of the proton-donor position is occupied by proline. Consequently the C-terminal region of the RLF B chain orients differently. Close contacts between the C-terminal region of the B chain and the A chain N-terminal and C-terminal region existing in insulin are absent in RLF. Even when a cross-linker forces the side chain of residue B26 toward the C-terminus of the A chain long-range NOEs between the two chains are not observed.

Like the fold of the insulin B-chain the fold of the RLF B chain is stabilized by hydrophobic interactions. In case of RLF this involves the indole ring in position (B27) and methyl groups that would otherwise expose a hydrophobic array on the surface of the B chain helix. This RLF-like B chain fold has been reported for human relaxin-3 (= insl7), where the relaxin-receptor (LGR7)-binding site becomes only accessible upon unfolding (15). The structural transition required for receptor-binding may not be universal for other relaxins as suggested by the x-ray structure of human relaxin-2 (14), however, the C-terminal B-chain sequence [CGGS(T)RW] in human relaxin 3 (9,15), tamar, shark and skate relaxins (59) suggests that the C-terminal structural motif is likely to persist in some relaxins. In rat, mouse, guinea pig, hamster, orangutan-II, rhesus monkey and baboon relaxin tryptophan(B27) is replaced by an aliphatic residue (59), which may not promote this fold. In general, the lack of solution structures and the extraordinary sequence diversity of relaxin (59) make it difficult to generalize.

While the hormones insulin, RLF, and relaxin-3 must go through a similar conformational transition in order to bind and activate their receptor, information on structural requirements of the corresponding receptor is still limited. Initial observations on ligand binding to LGR7 and LGR8 show some similarities to the better-investigated insulin tyrosine kinase receptor. Svendsen et al. (26) reported LGR8 aggregation and negative cooperative binding of RLF to LGR8 and suggested that for receptor activation RLF binds to the high affinity site on one receptor and to the low affinity site of a second receptor within the aggregate. Such a mechanism is well established for the activation of the insulin receptor (60,61) where the high affinity site of the insulin monomer is in contact with the fibronectin domain III (FnIII) and the low affinity site with the second leucine-rich repeat domain (L2) on the opposite monomer in the receptor dimer (61-63). In difference, the high affinity site on the RLF and relaxin receptors is located on a contiguous array on the concave surface of the leucine-rich repeat surface (27,64) whereas the low affinity site might be located on the 7-transmembrane domain (46).

In conclusion NMR data and the solution structure of a fully active cross-linked RLF derivative support that a transition of a compact non-binding conformer to a relaxed binding conformer occurs and that such a transition is a frequent mechanism by which hormones of the relaxin-insulin family interact with their corresponding receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The 800 MHz spectra were acquired at The Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules. We thank Jens Ø. Duus and Bent O. Petersen for technical assistance.

References

1. Ryle AP, Sanger F, Smith LF, Kitai R. The disulphide bonds of insulin. *Biochem J* 1955;60:541–556. [PubMed: 13249947]
2. Rinderknecht E, Humbel RE. The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *J Biol Chem* 1978;253:2769–2776. [PubMed: 632300]
3. Schwabe, C.; McDonald, JK. *Science*. Vol. 197. New York, N.Y.: 1977. Relaxin: a disulfide homolog of insulin; p. 914-915.
4. Isaacs N, James R, Niall H, Bryant-Greenwood G, Dodson GG, Evans A, North ACT. Relaxin and its structural relationship to insulin. *Nature* 1978;271:278–281. [PubMed: 622170]
5. Adham IM, Burkhardt E, Benahmed M, Engel W. Cloning of a cDNA for a novel insulin-like peptide of the testicular Leydig cells. *J Biol Chem* 1993;268:26668–26672. [PubMed: 8253799]
6. Chassin D, Laurent A, Janneau JL, Berger R, Bellet D. Cloning of a new member of the insulin gene superfamily (INSL4) expressed in human placenta. *Genomics* 1995;29:465–470. [PubMed: 8666396]
7. Conklin D, Lofton-Day CE, Haldeman BA, Ching A, Whitmore TE, Lok S, Jaspers S. Identification of INSL5, a new member of the insulin superfamily. *Genomics* 1999;60:50–56. [PubMed: 10458910]
8. Kasik J, Muglia L, Stephan DA, Menon RK. Identification, chromosomal mapping, and partial characterization of mouse insl6: A new member of the insulin family. *Endocrinology* 2000;141:458–461. [PubMed: 10614671]
9. Bathgate RAD, Samuel CS, Burazin TCD, Layfield S, Laasz AA, Reytomas IGT, Dawson NF, Zhao C, Bond C, Summers RJ, Parry LJ, Wade JD, Tregear GW. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene: Novel members of the relaxin peptide family. *J Biol Chem* 2002;277:1148–1157. [PubMed: 11689565]
10. Liu C, Eriste E, Sutton S, Chen J, Roland B, Kuei C, Farmer N, Joernvall H, Sillard R, Lovenberg TW. Identification of relaxin-3/INSL7 as an endogenous ligand for the orphan G-protein-coupled receptor GPCR135. *J Biol Chem* 2003;278:50754–50764. [PubMed: 14522968]
11. Blundell T, Cutfield JF, Cutfield SM, Dodson EG, Dodson GG, Hodgkin D, Mercola DA, Vijayan M. Atomic positions in rhombohedral 2-zinc insulin crystals. *Nature* 1971;231:506–511. [PubMed: 4932997]
12. Sato A, Nishimura S, Ohkubo T, Kyogoku Y, Koyama S, Kobayashi M, Yasuda T, Kobayashi Y. Three-dimensional structure of human insulin-like growth factor-I (IGF-I) determined by 1H-NMR and distance geometry. *Int J Peptide Protein Res* 1993;41:433–440. [PubMed: 8391516]
13. Torres AM, Forbes BE, Aplin SE, Wallace JC, Francis GL, Norton RS. Solution structure of human insulin-like growth factor II. Relationship to receptor and binding protein interactions. *J Mol Biol* 1995;248:385–401. [PubMed: 7739048]
14. Eigenbrot C, Randal M, Quan C, Burnier J, O'Connell L, Rinderknecht E, Kossiakoff AA. X-ray structure of human relaxin at 1.5 Å: Comparison to insulin and implications for receptor binding determinants. *J Mol Biol* 1991;221:15–21. [PubMed: 1656049]
15. Rosengren KJ, Lin F, Bathgate RAD, Tregear GW, Daly NL, Wade JD, Craik DJ. Solution structure and novel insight into determinants of receptor specificity of human relaxin-3. *J Biol Chem* 2006;281:5845–5851. [PubMed: 16365033]
16. Rosengren KJ, Zhang S, Lin F, Daly NL, Scott DJ, Hughes RA, Bathgate RA, Craik DJ, Wade JD. Solution structure and characterization of the receptor binding surface of insulin-like peptide 3. *J Biol Chem* 2006;281:28287–28295. [PubMed: 16867980]
17. Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M, Mason A, Seeburg P, Grunfeld C, Rosen OM, Ramachandran J. Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* 1985;313:756–761. [PubMed: 2983222]
18. Ebina Y, Ellis L, Jarnagin K, Edery M, Graf L, Clauser E, Ou JH, Masiarz F, Kan YW, Goldfine ID, Roth RA, Rutter WJ. The human insulin receptor cDNA: The structural basis for hormone-activated transmembrane signalling. *Cell* 1985;40:747–758. [PubMed: 2859121]
19. Ullrich A, Gray A, Tam AW, Yang-Feng T, Tsubokawa M, Collins C, Henzel W, Le Bon T, Kathuria S, Chen E, Jacobs S, Francke U, Ramachandran J, Fujita-Yamaguchi Y. Insulin-like growth factor I

- receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO J* 1986;5:2503–2512. [PubMed: 2877871]
20. Overbeek PA, Gorlov IP, Sutherland RW, Houston JB, Harrison WR, Boettcher-Tong HL, Bishop CE, AgoulNIK AI. A transgenic insertion causing cryptorchidism in mice. *Genesis* 2001;30:26–35. [PubMed: 11353515]
 21. Hsu, SY.; Nakabayashi, K.; Nishi, S.; Kumagai, J.; Kudo, M.; Sherwood, OD.; Hsueh, AJW. *Science*. Vol. 295. New York, N.Y.: 2002. Activation of orphan receptors by the hormone relaxin; p. 671–674.
 22. Liu C, Chen J, Sutton S, Roland B, Kuei C, Farmer N, Sillard R, Lovenberg TW. Identification of relaxin-3/INSL7 as a ligand for GPCR142. *J Biol Chem* 2003;278:50765–50770. [PubMed: 14522967]
 23. Adham IM, AgoulNIK AI. Insulin-like 3 signalling in testicular descent. *Int J Androl* 2004;27:257–265. [PubMed: 15379965]
 24. Büllesbach EE, Schwabe C. The primary structure and the disulfide links of the bovine relaxin-like factor (RLF). *Biochemistry* 2002;41:274–281. [PubMed: 11772026]
 25. Büllesbach EE, Schwabe C. Synthetic cross-links arrest the C-terminal region of the relaxin-like factor (RLF) in an active conformation. *Biochemistry* 2004;43:8021–8028. [PubMed: 15209497]
 26. Svendsen AM, Vrecl M, Ellis TM, Heding A, Kristensen JB, Wade JD, Bathgate RA, De Meyts P, Nohr J. Cooperative binding of insulin-like peptide 3 to a dimeric relaxin family peptide receptor 2. *Endocrinology* 2008;149:1113–1120. [PubMed: 18063691]
 27. Scott DJ, Wilkinson T, Zhang S, Ferraro T, Wade J, Tregear GW, Bathgate RA. Defining the LGR8 residues involved in binding insl3. *Mol Endocrinol* 2007;21:1699–1713. [PubMed: 17473281]
 28. Büllesbach EE, Schwabe C. The mode of interaction of the relaxin-like factor (RLF) with the leucine-rich repeat G protein-activated receptor 8. *J Biol Chem* 2006;281:26136–26143. [PubMed: 16844694]
 29. Büllesbach EE, Schwabe C. Tryptophan B27 in the relaxin-like factor (RLF) is crucial for RLF receptor binding. *Biochemistry* 1999;38:3073–3078. [PubMed: 10074360]
 30. Piotto M, Saudek V, Sklenar V. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J Biomol NMR* 1992;2:661–665. [PubMed: 1490109]
 31. Rance M, Sørensen OW, Bodenhausen G, Wagner G, Ernst RR, Wuthrich K. Improved spectral resolution in COSY ¹H NMR spectra of proteins via double quantum filtering. *Biochem Biophys Res Commun* 1983;117:479–485. [PubMed: 6661238]
 32. Jeener J, Meier BH, Bachmann P, Ernst RR. Investigation of exchange processes by two-dimensional NMR spectroscopy. *J Phys Chem* 1979;71:4546.
 33. Macura S, Huang Y, Suter D, Ernst RR. Two-dimensional chemical exchange and cross-relaxation spectroscopy of coupled nuclear spins. *J Magn Resonance* 1981;43:259–281.
 34. Braunschweiler L, Ernst RR. Coherence transfer by isotopic mixing application to proton correlation spectroscopy. *J Magn Resonance* 1983;53:521–528.
 35. Bax A, Davis DG. MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J Magn Resonance* 1985;65:355–360.
 36. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* 1995;6:277–293. [PubMed: 8520220]
 37. Wüthrich, K. *NMR of proteins and nucleic acids*. Wiley and Sons; New York: 1986.
 38. Wishart DS, Sykes BD, Richards FM. The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. *Biochemistry* 1992;31:1647–1651. [PubMed: 1737021]
 39. Baxter NJ, Williamson MP. Temperature dependence of ¹H chemical shifts in proteins. *J Biomol NMR* 1997;9:359–369. [PubMed: 9255942]
 40. Kristensen SM, Sørensen MD, Gesmar H, Led JJ. Estimation of signal intensities in 2D NMR spectra with severe baseline distortion by combined linear-prediction and least-square analyses. *J Magn Resonance B* 1996;112:193–193.
 41. Berjanskii MV, Neal S, Wishart DS. PREDITOR: a web server for predicting protein torsion angle restraints. *Nucleic Acids Res* 2006;34:W63–69. [PubMed: 16845087]

42. Barsukov, IL.; Lian, LY. Structure determination from NMR data. I Analysis of NMR data. In: Roberts, GCK., editor. NMR of macromolecules, A practical approach. IRL Press; Oxford: 1993. p. 315-358.
43. Rieping W, Habeck M, Bardiaux B, Bernard A, Malliavin TE, Nilges M. ARIA2: automated NOE assignment and data integration in NMR structure calculation. *Bioinformatics* (Oxford, England) 2007;23:381–382.
44. Schwieters CD, Kuszewski JJ, Tjandra N, Clore GM. The Xplor-NIH NMR molecular structure determination package. *J Magn Reson* 2003;160:65–73. [PubMed: 12565051]
45. Nilges M, O'Donoghue SI. Ambiguous NOE and automated NOE assignment. *Progr NMR Spectr* 1998;32:107–139.
46. Halls ML, Bond CP, Sudo S, Kumagai J, Ferraro T, Layfield S, Bathgate RA, Summers RJ. Multiple binding sites revealed by interaction of relaxin family peptides with native and chimeric relaxin family peptide receptors 1 and 2 (LGR7 and LGR8). *J Pharmacol Exp Ther* 2005;313:677–687. [PubMed: 15649866]
47. Baker EN, Blundell TL, Cutfield JF, Cutfield SM, Dodson EJ, Dodson GG, Crowfoot Hodgkin DM, Hubbard RE, Isaacs NW, Reynolds CD, Sakabe K, Sakabe N, Vijayan NM. The structure of 2Zn pig insulin crystals at 1.5Å resolution. *Phil Trans R Soc Lond B* 1988;319:369–456. [PubMed: 2905485]
48. Hua QX, Weiss MA. Comparative 2D NMR studies of human insulin and des-pentapeptide insulin: Sequential resonance assignment and implications for protein dynamics and receptor recognition. *Biochemistry* 1991;30:5505–5515. [PubMed: 2036420]
49. Chang X, Jorgensen AM, Bardrum P, Led JJ. Solution structures of the R6 human insulin hexamer. *Biochemistry* 1997;36:9409–9422. [PubMed: 9235985]
50. Bocian W, Sitkowski J, Bednarek E, Tarnowska A, Kawecki R, Kozerski L. Structure of human insulin monomer in water/acetonitrile solution. *J Biomol NMR* 2008;40:55–64. [PubMed: 18040865]
51. Zoete V, Meuwly M, Karplus M. A comparison of the dynamic behavior of monomeric and dimeric insulin shows structural rearrangements in the active monomer. *J Mol Biol* 2004;342:913–929. [PubMed: 15342246]
52. Mirmira RG, Tager HS. Role of the phenylalanine B24 side chain in directing insulin interaction with its receptor: importance of main chain conformation. *J Biol Chem* 1989;264:6349–6354. [PubMed: 2649499]
53. Weiss MA, Hua QX, Lynch CS, Frank BH, Shoelson SE. Heteronuclear 2D NMR studies of an engineered insulin monomer: Assignment and characterization of receptor-binding surface by selective ²H and ¹³C labeling with application to protein design. *Biochemistry* 1991;30:7373–7389. [PubMed: 1906742]
54. Hua QX, Shoelson SE, Kochoyan M, Weiss MA. Receptor binding redefined by a structural switch in a mutant human insulin. *Nature* 1991;354:238–241. [PubMed: 1961250]
55. Hua QX, Kochoyan M, Weiss MA. Structure and dynamics of des-pentapeptide-insulin in solution: the molten-globule hypothesis. *Proc Natl Acad Sci USA* 1992;89:2379–2383. [PubMed: 1549601]
56. Jørgensen AMM, Olsen HB, Balschmidt P, Led JJ. Solution structure of the superactive monomeric des-[Phe(B25)] human insulin mutant - elucidation of the structural basis for the monomerization of des-[Phe(B25)] insulin and the dimerization of native insulin. *J Mol Biol* 1996;257:684–699. [PubMed: 8648633]
57. Keller D, Clausen R, Josefsen K, Led JJ. Flexibility and bioactivity of insulin: an NMR investigation of the solution structure and folding of an unusually flexible human insulin mutant with increased biological activity. *Biochemistry* 2001;40:10732–10740. [PubMed: 11524020]
58. Nakagawa SH, Hua QX, Hu SQ, Jia W, Wang S, Katsoyannis PG, Weiss MA. Chiral mutagenesis of insulin. Contribution of the B20-B23 beta-turn to activity and stability. *J Biol Chem* 2006;281:22386–22396. [PubMed: 16751187]
59. Schwabe, C.; Büllesbach, EE. *Relaxin and the fine structure of proteins*. Springer; New York: 1998. p. 13-23.
60. De Meyts P, Bianco AR, Roth J. Site-Site interactions among insulin receptors: Characterization of the negative cooperativity. *J Biol Chem* 1976;251:1877–1888. [PubMed: 5434]
61. De Meyts P. The insulin receptor: a prototype for dimeric, allosteric membrane receptors? *Trends Biochem Sci* 2008;33:376–384. [PubMed: 18640841]

62. McKern NM, Lawrence MC, Streltsov VA, Lou MZ, Adams TE, Lovrecz GO, Elleman TC, Richards KM, Bentley JD, Pilling PA, Hoyne PA, Cartledge KA, Pham TM, Lewis JL, Sankovich SE, Stoichevska V, Da Silva E, Robinson CP, Frenkel MJ, Sparrow LG, Fernley RT, Epa VC, Ward CW. Structure of the insulin receptor ectodomain reveals a folded-over conformation. *Nature* 2006;443:218–221. [PubMed: 16957736]
63. Ward C, Lawrence M, Streltsov V, Garrett T, McKern N, Lou MZ, Lovrecz G, Adams T. Structural insights into ligand-induced activation of the insulin receptor. *Acta physiologica (Oxford, England)* 2008;192:3–9.
64. Büllesbach EE, Schwabe C. The trap-like relaxin-binding site of the leucine-rich G-protein-coupled receptor 7. *J Biol Chem* 2005;280:14051–14056. [PubMed: 15695505]

Abbreviations used

ARIA	Ambiguous Restraints for Iterative Assignment
DQF-COSY	Double Quantum Filtered - Correlation Spectroscopy
GPCR	G-protein coupled receptor
HSQC	heteronuclear single-quantum coherence
INSL3	insulin-like3
LGR8	leucine-rich G-protein coupled receptor-8
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
RLF	relaxin-like factor
hRLF	human relaxin-like factor
TOCSY	total correlation spectroscopy
WATERGATE	Water suppression through gradient tailored excitation

Fig.1.

Sequential- and medium-range NOE connectivities in human RLF (A) and the cross-linked RLF (B). The cross-linker consists of lysine replacing arginine in position B26 and an iso-peptide bond between the lysine side chain amino group and the carboxyl group of the glycine extension of the A chain. Glycine in position A27 serves as a spacer. The intensity of the NOE connectivities is indicated by the thickness of the bars: a distance of $<3\text{\AA}$ (thick), $3-4\text{\AA}$ (medium) and $>4\text{\AA}$ (thin).

Fig.2.

Chemical shift changes upon dilution of hRLF (pH 5.0; 35 °C). $\Delta[\text{ppm}] = \text{CS}_{1.2 \text{ mM}} - \text{CS}_{0.2 \text{ mM}}$. A) Amide protons of the A-chain, B) amide protons of the B chain, C) alpha protons of the A chain, D) alpha protons of the B chain. Glycine HA2 and HA3 were averaged. * No data.

Fig.3.

Display of short-range NOEs (sequence separation 2-3 residues), medium-range NOEs (sequence separation 4-5 residues), long-range NOEs (sequence separation >5 residues). NOEs between Gly(A27) and Lys(B26) were considered sequential. A) hRLF A chain, B) hRLF B chain, C) cross-linked RLF A chain, D) cross-linked RLF B chain.

Fig. 4.

Chemical shift differences between hRLF and cross-linked hRLF both at 1.2 mM concentration at pH 5.0 at 35 °C [$\Delta\text{ppm} = \text{CS}_{\text{hRLF}} (\text{ppm}) - \text{CS}_{\text{cross-linked}} (\text{ppm})$]. A) Backbone amide protons of the A chain, B) backbone amide protons of the B chain, C) alpha protons of the A chain, D) alpha protons of the B chain. Glycine HA2 and HA3 were averaged. * No data.

Fig. 5. Proton Chemical Shift indices of hRLF (black bars) cross-linked hRLF (red bars), Panel A: A chain residues, panel B: B chain residues. Asterisks indicate no or incomplete data.

Fig. 6. Temperature coefficients for the backbone amides of hRLF (black) and cross-linked RLF (red). Temperature coefficients less negative than -4.25 ppb/K (line) imply the existence of hydrogen bonds. Panel A: A chain residues, panel B: B chain residues.

Fig. 7. Comparison of hRLF and crosslinked RLF. Panels A/B view of hRLF(A) and cross-linked RLF (B) from the surface of the B chain helix. Panels C/D view of hRLF(A) and cross-linked RLF (B) through the B chain helix. Blue = B-chain helix, yellow = A-chain helices, red = Arg (B16), green = Val(B19), magenta = position B26 in hRLF and positions B26-A27 in cross-linked RLF, cyan = Trp(B27), N marks an N-terminus, C marks a C-terminus.

Fig. 8. Regions of the TOCSY spectra of hRLF (upper panel) and cross-linked RLF (lower panel) showing the chemical shifts and the multiplicity of the aromatic cross-peaks for TrpB27. W = TrpB27 major form and W' = TrpB27 minor form.

Fig. 9. Regions of the TOCSY spectra of hRLF and cross-linked RLF showing the chemical shifts and the multiplicity of the HA-HG cross-peaks for ValB15, ValB19, and ValB21.

Fig. 10.

Stereo view of the superposed 20 lowest energy structures of human RLF (blue) and cross-linked RLF (red). The cross-link is colored green and the cystine bonds are colored cyan for hRLF and black for cross-linked RLF. N-A and N-B represent the N-termini of the A chains and B chains, respectively. C-A and C-B label the C-termini of the A and B chains, respectively.

Table 1

NMR statistics for structural ensembles of hRLF and cross-linked RLF. The evaluation is based on the 20 lowest energy structures.

	hRLF	Cross-linked RLF
NOEs	1025	942
unambiguous	913	828
Intra-residue	412	392
Inter-residue		
Sequential (1)	202	176
Short range (2-3)	109	76
Medium range (4-5)	53	41
Long range (>5)	137	143
ambiguous	82	108
2 resonances	73	101
3 resonances	7	6
4 resonances	2	1
Total	995	936
H-bonds	13	13
³ J-coupling	21	
Dihedral angle restraints		
Phi	22	25
Psi	19	18
Chi	12	8
Violations		
NOEs and H-bonds (>0.5Å)	0.2	0.6
dihedral angles (>5 deg)	1.60	4.15
J coupling (>1 Hz)	0.65	-
RMSD (mean ±SD)		
NOEs (Å)	0.053 (±0.0015)	0.048 (±0.0075)
Bond length (Å)	0.0056 (±0.0002)	0.0042 (±0.0019)
Bond angle (deg)	0.73(±0.02)	0.62 (±0.028)
Improper (deg)	0.54 (±0.025)	0.40 (±0.028)
Average pair wise rmsd		
Helix A6-11 backbone (Å)	0.39 (±0.26)	0.42 (±0.039)
Helix A12-23 backbone (Å)	0.14 (±0.04)	0.44 (±0.11)
Helix B13-21 backbone (Å)	0.14 (±0.04)	0.28 (±0.04)
All backbone atoms (Å)	1.92 (±0.37)	0.95 (±1.25)
Ramachandran analysis ^a		
most favored (%)	69.8 (±5.8)	75.5 (±5.1)
allowed (%)	24.2 (±5.7)	21.7 (±4.7)
generously allowed (%)	3.2 (±1.8)	0.96 (±1.2)
disallowed (%)	2.8 (±2.91.9)	2.0 (±1.6)

^a After refinement in water.