Tryptophan B27 in the Relaxin-like Factor (RLF) Is Crucial for RLF Receptor-Binding†

Erika E. Büllesbach and Christian Schwabe*

Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, South Carolina 29425

Received November 11, 1998; Revised Manuscript Received December 23, 1998

ABSTRACT: The relaxin-like factor (RLF) is a circulating hormone that is synthesized in the gonads of mammals and released into the bloodstream. The distribution of its receptor and a trace of cross-reactivity to relaxin receptors implied that this relatively new factor is more relaxin- than insulin-like. The chemical synthesis of RLF analogues with specific modifications in positions B27 and B25, or the truncated form des(B27−31)RLF, clearly indicate that the intact indole ring in position B27 is crucial for high RLF receptor-binding. Receptor-binding was reduced by 2 orders of magnitude for Leu(B27)RLF (3%), Ala-(B27)RLF (2.1%), and des(B27−31)RLF (0.4%), whereas slightly better binding was observed for His-(B27)RLF (7.5%), Phe(B27)RLF (21%), D-Trp(B27) (26%), and the oxindole(B27)RLF (41%). On the basis of these observation it seems that an aromatic ring system in the β- or γ-position is required for binding. Structure prediction of the C-terminal region of the B chain indicated a possible type I or type III turn for residues C−G−G−P−R (B22−26) preceding the tryptophan. Exchanging Pro(B25) for D-Pro within the turn caused a severe structural rearrangement at the C terminus and a 96% drop in activity. It appears that the steric effect of L-Pro(B25) is important for the proper positioning of Trp(B27).

The relaxin-like factor (RLF), also known as Leydig insulin-like peptide (Ley I-L), is produced in gonadal tissues, in particular in testicular Leydig cells and the luteal cells of the ovary *(1−5)*. In males the physiological role of RLF appears to be on spermatogenesis and germ-cell maturation *(5)*. In ovaries expression coincides with follicle development and subsequent changes in expression levels during cycling and pregnancy *(5)*. In humans RLF is a circulating hormone with the highest levels detected in the serum of postpuberty males, while in females and children the RLF concentration is lower by 1 order of magnitude (unpublished results).

A potential target of RLF is the reproductive tract of both sexes. While the receptor-bearing tissue of the male is not known, in the female RLF binds specifically to cell membrane receptors. Mouse uterus and brain, for example, show specific, competitive binding of RLF tracer *(6)*. Both tissues are also targeted by relaxin, a hormone of reproduction *(7)*. In fact there is a weak cross-reactivity between RLF and relaxin and their respective receptors *(6)*. It was proposed that RLF cross-reacts to the relaxin receptor through two arginine side chains on the B chain helix *(8)*. In RLF this relaxin receptor-binding site, however, is offset by one helix turn *(6)*. RLF interacts with its own receptor most likely through a different site. It is our goal to define the active site of RLF, and in the present paper we show that the C-terminal region of the B chain, in particular tryptophan B27, is important for the high-affinity binding of RLF to its receptor.

MATERIALS AND METHODS

Amino acid derivatives were purchased from either Bachem Bioscience or Perkin-Elmer Applied Biosystems. Chemicals for peptide synthesis were obtained from Perkin-Elmer Applied Biosystems. Solvents for synthesis and chromatography were Burdick and Jackson B & J Brand high-purity solvents. All other chemicals were of ACS grade and were used without further purification. Human RLF was chemically synthesized and labeled with ^{125}I by the chloramine T method *(6)*.

High-Performance Liquid Chromatography (HPLC[1]*).* The mobile phase of all HPLC systems consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in 80% acetonitrile (solvent B).

Preparative HPLC was performed on a Dynamax HPLC dual pump system equipped with a Dynamax column (C18, 8 μm (41.4 × 250 mm); Rainin Instrument Co. Inc.). About 0.1−1 g of peptide was purified at a flow rate of 40 mL/min and the effluent detected by UV absorbance at 280 nm. Fractions of 10 mL were collected, and each individual fraction was evaluated by analytical HPLC.

For semipreparative HPLC a Waters dual pump HPLC system equipped with a Synchropak RP-P column (C18, 10 × 250 mm) was used. About 1−10 mg of peptide was separated at a flow rate of 3 mL/min. Peptides were detected by UV absorbance at 226 nm and collected manually.

Analytical HPLC (system 1) was accomplished using a Waters dual pump HPLC system in combination with a Bakerbond (C18, wide bored) column. About 10−50 μg of peptide was automatically injected and separated at a flow rate of 1 mL/min. Linear gradients from 20% to 60% B over

† This work was supported by NIH Grant GM 48893-9.

[1] Abbreviations: Acm, acetamidomethyl; Boc, *tert*-butyloxycarbonyl; But, *tert*-butyl; Fmoc, 9-fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; MALDI-MS: matrix-assisted laser desorption/ionization mass spectrometry; Tris, tris(hydroxymethyl)-aminomethane.

10.1021/bi982687u CCC: $18.00
Published on Web 02/17/1999

30 min were used and the effluents detected by UV absorbance at 220 nm.

For analytical HPLC (system 2) we used an ABI model 130A (Applied Biosystems) equipped with an Aquapore 300 column (C8, 2.1 × 30 mm). Approximately 1−2 μg of peptide were injected automatically and separated at a flow rate of 100 μL/min. Usually 60 min gradients were employed, and UV absorbance of the effluent was recorded at 230 nm.

Synthesis. RLF analogues were produced by solid-phase peptide synthesis using differential protection for the thiol groups for the specific site-directed formation of the disulfide bonds. Two different chemical routes were employed: The first route is essentially described in the literature *(6)* using Boc chemistry for the B chain and Fmoc chemistry for the A chain.

The second method is based on Fmoc chemistry for both chains. For trifunctional amino acids the following side chain-protecting groups were chosen: *tert*-butyl ether for Ser, Thr, and Tyr, *tert*-butyl ester for Asp and Glu, *tert*-butyloxycarbonyl for Lys, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for arginine, sulfoxide for methionine, trityl for His, Asn, Gln, and CysA10, CysA15, and CysB22, acetamidomethyl for CysA11 and CysB10, and *tert*-butyl for CysA24. The tryptophan side chain was either Boc-protected or remained unprotected during the synthesis. The C-terminal amino acid was condensed to Wang resin and the peptide assembled by utilizing a Perkin-Elmer Applied Biosystems automatic synthesizer (model 433A for 0.25 mmol synthesis and model 432A for 25 μmol synthesis). The final peptidyl resins were deprotected with trifluoroacetic acid−phenol−ethylenedithiol−thioanisole−water (10:0.75:0.25:0.5:0.5, v/w/v/v/v) *(9)* for 2 h at room temperature. The resin was filtered off and the peptide precipitated with ether. After centrifugation the pellet was washed with ether three times, thereafter the pellet was air-dried, suspended in 5 mL of water, and lyophilized.

Preparation of S-Acetamidomethyl(A11), S-2-Pyridylsulfenyl(A24) A Chain Disulfide(A10,15). The crude A chain (AcmA11, ButA24) was dissolved in 50% acetic acid at a peptide concentration of 10 mg/mL. Iodine (50 mM in acetic acid) was slowly added until a slight yellow color remained. Excess iodine was reduced with 1 M ascorbic acid in water, and the reaction mixture was diluted to 17% acetic acid and separated by preparative HPLC. The column was equilibrated with 20% B and, after unbound UV-absorbing material was removed under equilibration conditions, a 30 min linear gradient from 20% to 70% B was employed. Pure A chain-containing fractions were pooled and lyophilized (yield 182.4 mg, 25.1% based on the first amino acid bound to the resin).

The *tert*-butyl thioether group in position A24 was converted to the 2-pyridylsulfenyl group in analogy to the procedure described by Maruyama et al. *(10)*. RLF A chain (AcmA11, ButA24) (49.8 mg, 17.16 μmol) and 2,2′-dipyridyl disulfide (53 mg, 241 μmol) were dissolved in 0.9 mL of trifluoroacetic acid containing 100 μL of thioanisole. The mixture was chilled on ice, 1 mL of trifluoromethanesulfonic acid/trifluoroacetic acid (1:4 v/v) was added, and the reaction was stirred for 30 min at 0 °C. The peptide was precipitated with 8 mL of chilled ether and collected by centrifugation. The pellet was washed 3 times with 10 mL of ether. The air-dried pellet was suspended in 3 mL of 5% ammonium acetate acidified with glacial acetic acid (final concentration approximately 20%), desalted on Sephadex G25 sf in 1 M acetic acid, and lyophilized. Portions of 10 mg were HPLC purified on Synchropak RP-P (C_{18} 10 × 250 mm), using a linear gradient from 30 to 50% B over 30 min, and lyophilized. Yield: RLF A chain (SS, Acm, SPy) 38.6 mg (77.5%).

RLF B Chains with Replacements of Trp(B27). The C-terminal peptide (B28−31) Fmoc-Ser(But)-Thr(But)-Glu-(OBut)-Ala-resin was prepared in 0.25 mmol scale; the resin was divided into four aliquotes which were used to incorporate either His, Ala, Leu, or Phe into position B27. Thereafter the resins were recombined and the synthesis was continued on a 0.25 mmol scale. All peptides were deprotected and purified on Dynamax C_{18} using a linear gradient from 16% to 56% B over 30 min. Fractions containing a single peak in analytical HPLC were lyophilized and identified by amino acid analysis [yields: His(B27), 54.6 mg (24.3%); Ala(B27), 46 mg (20.4%); Leu(B27), 57 mg (25.3%); Phe(B27), 39.2 mg (17.4%)]. Amino acid analyses are as follows: RLF, Ala 6, Leu 6, His 2, Phe 1; Ala(B27)-RLF, 6.73 (7); Leu 6.05 (6); His 2.20 (2), Phe 0.97 (1); Leu-(B27)RLF, Ala 6.00 (6), Leu 6.96 (7), His 2.13 (2), Phe 0.99 (1); Phe(B27)RLF, Ala 6.00 (6), Leu 6.29 (6), His 2.02 (2), Phe 1.81 (2); His(B27)RLF, Ala 5.92 (6), Leu 6.02 (6), His 3.17 (3), Phe 0.97 (1).

Formation of the Interchain Disulfide Link A24/B22. The *S*-pyridylsulfenyl A chain (12.9 mg, 4.37 μmol) and monothiol B chain 16.3 mg, 4.53 μmol) were dissolved in 3 mL of 0.1 M acetic acid containing 8 M guanidinium chloride (pH 4.5) and stirred at 37 °C for 24 h. The combined product was purified on Sephadex G50 sf (column 2.5 cm × 55 cm) in 1 M acetic acid, followed by semipreparative HPLC using a linear gradient of 25% to 50% B over 30 min. Yields: 45−72%.

Formation of the Third Disulfide Bond. Removal of the Acm groups was carried out with iodine in aqueous acetic acid. RLF analogues without tryptophan were reacted in 70% acetic acid *(6, 11)*, and unprotected tryptophan-containing RLF analogues were reacted in 95% acetic acid *(10)*. After excess iodine was reduced with aqueous ascorbic acid the peptide was desalted on Sephadex G25 sf (2.5 × 50 cm) and purified by semipreparative HPLC employing a linear gradient from 25% to 50% B over 30 min (yields 25−45%).

Reduction of Methionine Sulfoxide. RLF−sulfoxide(B5) (7.9 mg, 1.25 μmol) was reduced with 800 μL of 0.05 M NH_4I in trifluoroacetic acid/water (9:1 v/v) for 15 min on ice *(12, 13)*, and the reaction was quenched with 3 mM ascorbic acid in 5 mL of chilled water and immediately separated by semipreparative HPLC using a linear gradient from 25% to 50% B over 30 min. Yields: 40−75%.

Oxidation of Tryptophan in RLF with N-Bromosuccinimide. RLF (0.278 mg), dissolved in 800 μL of 0.2 M acetate buffer (pH 4.7), was transferred into a 1.5 mL quartz cuvette. *N*-Bromosuccinimide (10 mM in 0.2 M acetate buffer pH 4.7) was added in increments of 2 μL, and UV spectra were recorded after each addition. In parallel porcine relaxin as a nontyrosine containing standard protein was reacted. On the basis of this standard reaction, 9 μL of *N*-bromosuccinimide was sufficient to completely oxidize the tryptophan in RLF. The oxidized RLF sample was used for assay without isolation.

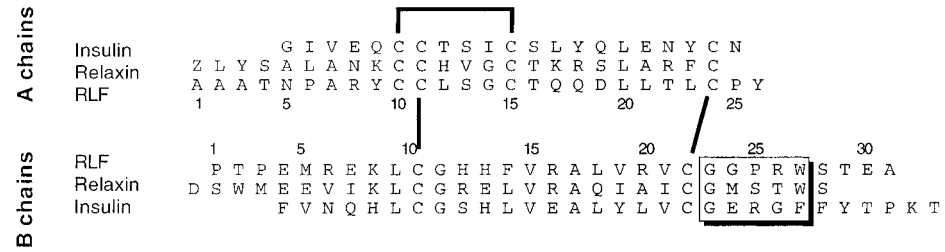

FIGURE 1: Primary structure of human RLF compared with that of human insulin and human relaxin II. The boxed-in region includes the RLF region which might include the receptor-binding site (Z = pyroglutamine)

Analytic: *Reduction and Chain Separation*. About 10 μg of the RLF analogue in 20 μL of water was reduced with 20 μL of 50 mM dithiothreitol solution in 6 M guanidinium chloride and 0.2 M Tris/HCl at pH 8.6. The reduction was completed in 1 h at 37 °C, the reaction acidified with acetic acid (5 μL), and about 10 μL injected for analytical HPLC (system 2), using a linear gradient from 20 to 35% B over 60 min.

Amino Acid Analysis. Peptides were hydrolyzed in vapor phase 6 M HCl containing 0.1% phenol for 1 h at 150 °C. The amino acids were detected after precolumn modification with phenyl isothiocyanate and separated by HPLC (Pico·Tag system, Waters Millipore).

Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry. RLF analogues were dissolved in 0.1% trifluoroacetic acid in 40% acetonitrile at a concentration of 1 μg/μL. An aliquot was mixed with 50 mM α-cyano-4-hydroxycinnamic acid (1:3 v/v), and 1 μL was placed on a sample probe and air-dried. Mass spectra were acquired on a Voyager-DE Biospectrometry Workstation (Perseptive Biosystems) at the Mass Spectrometry Facility at MUSC.

Ultraviolet Spectroscopy. In a parallel experiment RLF dissolved in water (concentration approximately 1.5 mg/mL) was diluted either with water (1:3 v/v) or with 8 M guanidinium chloride containing 0.1 M acetic acid/NaOH at pH 4.5 and 1:3 v/v. When measured in water, the UV absorbance was suppressed by 4%. The same experiment was performed with Phe(B27)RLF. UV absorbance in water was suppressed by less than 5%. Subsequently, the protein contents of all RLF analogues were determined in water using the theoretical calculated specific absorbance coefficients, 1.31 for Trp containing RLF and 0.43 for analogues lacking tryptophan.

Circular Dichroism. The RLF stock solutions (0.4−0.8 mg/mL) in water were diluted with the same volume of 50 mM Tris/HCl buffer at pH 7.5. Far-UV circular dichroism (CD) spectra were acquired using a Jasco J710 spectrapolarimeter at a resolution of 0.2 nm, with a bandwidth of 2 nm. Ten spectra were averaged. Molar ellipticity was calculated according to the literature *(14)*.

Receptor-Binding Assays. Virgin female ICR mice (Charles River) were primed with 5 μg of estrogen cypionate in 100 μL of sesame oil. Five days later the mice were killed in an atmosphere of CO_2, the uteri excised, and four uteri dropped into 15 mL of chilled homogenizing buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 8 mg/L soybean trypsin inhibitor, pH 7.5) supplemented with 0.25 M sucrose. The tissue was homogenized on ice for 10 s with a Polytron homogenizer at setting 7. The homogenate was centrifuged at 700 rpm for 10 min at 4 °C. The pellet was resuspended in 10 mL of homogenizing buffer with sucrose addition, homogenized again, and centrifuged at 700 rpm for 10 min at 4 °C. The supernatants of the two low-speed centrifugations were combined and centrifuged at 20 000*g* for 1 h at 4 °C. The pellet was resuspended in homogenizing buffer without sucrose addition and centrifuged at 20 000*g* for 1 h at 4 °C. The supernatant was discarded and the pellet resuspended in 1 mL of binding buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 25 μM $MgCl_2$, 1.5 mM $MnCl_2$, 1.6 mM $CaCl_2$, 1% bovine serum albumin, and 0.2 mM phenylmethylsulfonyl fluoride, pH 7.5).

Receptor-binding assays were performed in 1.5 mL Eppendorf vials using ^{125}I-labeled RLF *(6)* (40 μL, 100 000 cpm, 25 fmol per assay), RLF or RLF analogues at various concentrations (20 μL), and crude membranes (40 μL). The assay was incubated for 1 h at room temperature. Thereafter the suspension was diluted with 1 mL of chilled wash buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 1% bovine serum albumin, 0.01% NaN_3) and centrifuged at 14 000 rpm in an Eppendorf microcentrifuge for 10 min at room temperature. The supernatant was discarded and the tip of the vial cut and counted in a γ-counter. Nonspecific binding was determined in the presence of 20 μg/mL (3170 nM) unlabeled RLF, and total binding was determined in the absence of RLF. In a typical experiment the specific binding was between 45% and 60% of the total binding. Each datapoint was determined in duplicate. At least three independent dose response curves were acquired for each RLF analogue and compared with unmodified RLF run in parallel. The data were averaged and the dose response curves were fitted as described by De Lean et al. *(15)* and evaluated and displayed using KaleidaGraph.

RESULTS AND DISCUSSION

Analogues of the relaxin-like factor (Figure 1) were synthesized using different protecting groups for the cysteines that allowed for a site-directed formation of the disulfide bonds. These syntheses require the removal of acetamidomethyl groups by treatment with high excess of iodine in the presence of the readily oxidized free side chain of tryptophan *(16)*. RLF contains only one tryptophan which, if replaceable by another natural amino acid, would ease the synthesis of other derivatives. Phenylalanine would be the most conservative change in terms of aromaticity, histidine would best mimic the pyrrole ring of indole and would still permit hydrogen bond formation, and alanine as the smallest chiral amino acid would function as spacer, whereas leucine would cause a more drastic change in space requirement by replacing a planar aromatic structure with a bulky isopropyl group. The four different B chains were synthesized together,

Table 1: MALDI Mass Spectrometry of RLF Analogs

RLF analog	MH^+(found)	MH^+(calcd)
RLF	6293.8	6293.2
Ala(B27)	6176.9	6178.1
His(B27)	6244.3	6244.1
Leu(B27)	6218.0	6220.2
Phe(B27)	6255.0	6254.4
D-Trp(B27)	6292.9	6293.2
Ala(B25)	6266.6	6267.2
D-Pro(B27)	6292.2	6293.2
des(B27−31)	5719.0	5718.6

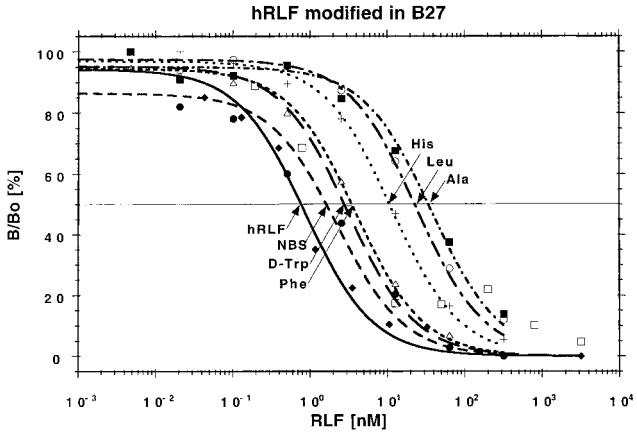

FIGURE 2: Receptor-binding assays of RLF analogues modified in position B27. Crude membranes of estrogen-primed mouse uteri (4 mice/assay) were used in combination with ^{125}I-labeled RLF. The concentrations of half-maximal response are 0.75 nM for (◆) RLF (solid line), 1.8 nM for (●) *N*-bromosuccinimide-treated RLF, 2.9 nM for (□) D-Trp(B27)RLF, 3.5 nM for (△) Phe(B27)RLF, 10 nM for (+) His(B27)RLF, 25 nM for (○) Leu(B27)RLF, and 35 nM for (■) Ala(B27)RLF. Nonspecific binding was determined in the presence of 3.17 μM RLF. All assays were performed in duplicate, and three independent assays were averaged. The curves were fitted according to deLean et al. *(15).* (NBS: human RLF treated with *N*-bromosuccinimide.)

and after deprotection, the individual B chains were separated by HPLC and subsequently each combined with an A chain yielding four RLFs with different substitutions in position B27. Homogeneity of the analogues was verified by analytical HPLC in two systems. After reduction, followed by HPLC separation, two chains were detected. The substituent in position B27 was verified by amino acid analysis, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Table 1), and protein sequencing of the Leu(B27)-RLF.

The relative affinity of the B27-substituted RLFs was determined by competitive binding of ^{125}I-labeled RLF to RLF receptor-bearing crude membrane preparations of mouse uteri. The assay results (Figure 2) implied that replacing tryptophan in B27 by any amino acid reduced the affinity by almost 2 orders of magnitude for both aliphatic amino acids (Ala, Leu) whereas Phe(B27) and His(B27) retained 21% and 7.5% of the RLF binding. A distinct drop in activity was also observed when RLF was oxidized with *N*-bromosuccinimide, a tryptophan-specific reagent leading to oxindole formation but not to peptide bond cleavage *(17)*. This implied that the indole ring was essential for proper RLF receptor-binding. Replacement of L-Trp by the corresponding D-enantiomer, D-Trp(B27)RLF, left us with an RLF that was only slightly more active than Phe(B27)RLF. This observation suggests that the indole ring is better than everything

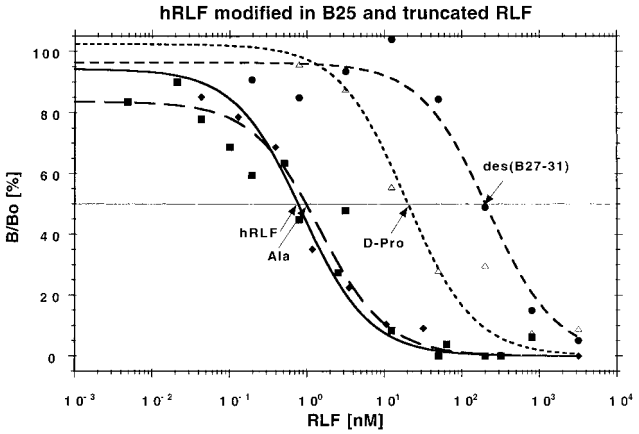

FIGURE 3: Receptor-binding assays of RLF analogues modified in position B25 and RLF truncated at the C terminus of the B chain, des(B27−31)RLF. Crude membranes of estrogen-primed mouse uteri (4 mice/assay) were used in combination with ^{125}I-labeled RLF. The concentrations of half-maximal response are 0.75 nM for (◆) RLF (solid line), 1.0 nM for (■) Ala(B25)RLF, 20 nM for (△) D-Pro(B25)RLF, and 210 nM for (●) desB(27−31)RLF. Nonspecific binding was determined in the presence of 3.17 μM RLF. All assays were performed in duplicate, and three independent assays were averaged and fitted according to deLean et al. *(15).*

else even if it is presented at a different angle. The most inactive analogue which retained only 0.4% of control was obtained when RLF was truncated at the C terminus of the B chain (des-B(27−31)RLF) (Figure 3).

If the region is sensitive to the structure, it should not be just a tryptophan effect. The conserved residues CGGPRWS may form a β-turn, and disturbance of this motif would change the relative position of the indole side chain. To eliminate the structural restriction of proline(B25) two analogues were designed which would either increase the flexibility (replacing Pro by Ala) or change the orientation (by introducing D-Pro). Ala(B25)RLF showed an affinity for the receptor similar to human RLF (75%), while D-Pro(B25) reduced binding to 3.8% (Figure 3). This observation indicates that a more flexible Ala(B25)RLF can assume the active conformation while the sterically restricted D-Pro-(B25)RLF moves the C-terminal end into an unfavorable position.

The far-UV CD spectra of the analogues and of human RLF showed the typical shape of a helical protein with a minimum at 208 nm and a maximum at 195 nm with a crossover at 200 nm. The CD spectra of all derivatives showed reduced intensity when compared to RLF (Figure 4A−C). There is no correlation between receptor-binding abilities of the analogues and their secondary structure. In particular Ala(B25)RLF and D-Pro(B25)RLF showed identical CD spectra (Figure 4C), but receptor-binding varied from 75% for Ala(B25)RLF to 3.8% for D-Pro(B25)RLF. All derivatives in which Trp(B27) is replaced by either L- or D-amino acids resulted in the same crossover at 200 nm as observed for RLF. A small red-shift was registered for des-(B27−31)RLF and both B25 modified analogues.

Although the structure of circulating RLF is unknown, it is presumably a two-chain molecule consisting of a 26 residue A chain and a 31 residue B chain *(2, 18)*. Homology between the primary structures of RLF and other hormones is low, ranging from 36% for human relaxin II to 34% for human insulin. The secondary structure could be predomi-

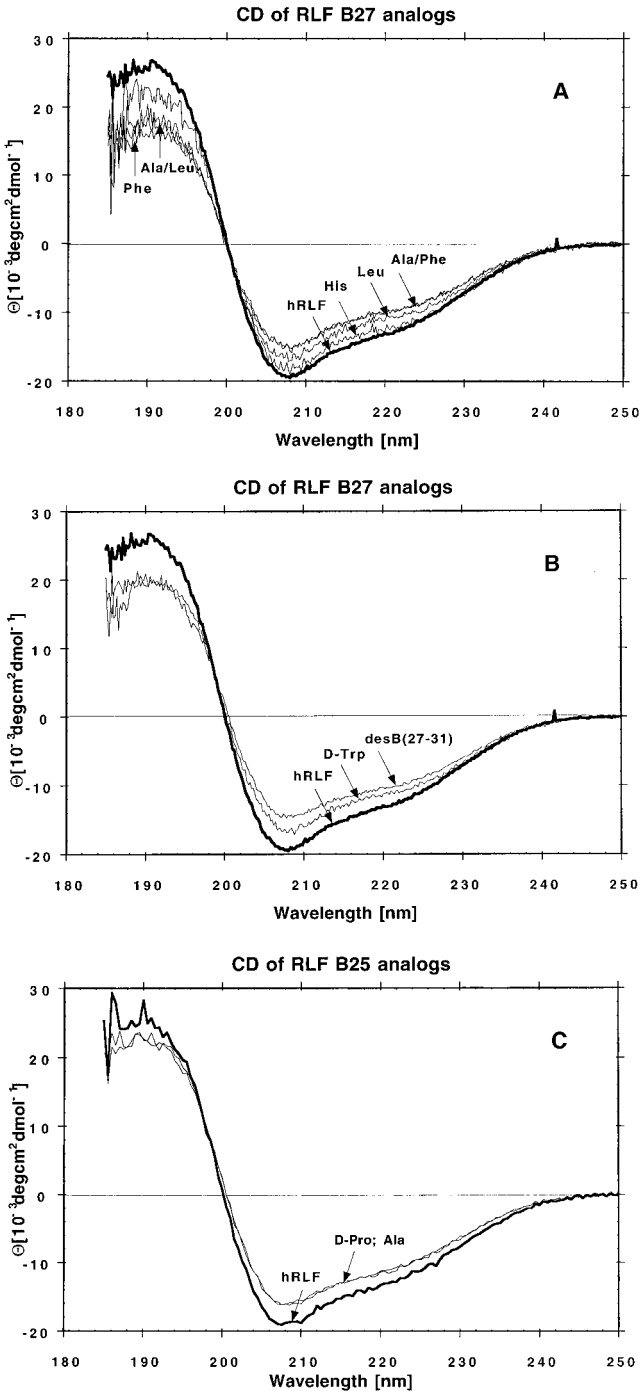

FIGURE 4: Far-UV circular dichroism of RLF and analogues. Proteins at concentrations of 0.2−0.4 mg/mL were measured in 25 mM Tris/HCl (pH 7.5) in a 0.2 mm cylindrical cell. Data were collected at a resolution of 0.2 nm with a bandwidth of 2 nm, and 10 spectra were averaged. (A) RLF analogues modified in position B27. The bold solid line is hRLF, and the thin solid lines are analogues in which B27 is replaced by His, Leu, Ala, or Phe. The signal intensity of the analogues is reduced over RLF and decreased in the order His(B27) > Leu(B27) > Ala(B27) ∼ Phe(B27). (B) RLF analogues D-Trp(B27) and des(B27−31)RLF showing a reduced signal intensity when compared to RLF (thick solid line). DesB(27−31)RLF shows a small redshift of the crossover. (C) RLF analogues modified in position B25. Both, D-Pro(B25) and Ala-(B25)RLF, are identical and show a lower signal when compared to RLF (thick solid line). Both analogues show a small red shift of the crossover.

nantly helical, most likely an amphiphilic helix in the midregion of the B chain and the C-terminal region of the A chain. The N-terminal segment of the A chain is probably not a continuous helix because of the asparaginyl−prolyl (A5−A6) sequence.

The C-terminal region of the B chain is ill-defined. Building the structure on the insulin coordinates *(19)* suggests a conflict between the extended N terminus of the A chain and a β-sheet in the C-terminal region of the B chain. Therefore, the orientation of the C-terminal region of the B chain (B23−31) should differ from that of the same region in insulin. In addition, the glycine distribution in the C-terminal region of RLF and insulin are different which results in a different orientation. The RLF structure built on the X-ray structure of human relaxin *(20)* would not be informative because the C-terminal region of the relaxin B chain is too short and too flexible. Even though the sequence C−G−G−P (B22−25) restricts the RLF conformation to either a type I or type II turn or possibly a 3_{10} helix *(21)*; our data support the hypothesis that this region of the RLF structure belongs to the receptor-binding locus.

ACKNOWLEDGMENT

The authors thank Robert Bracey, Barbara Rembiesa, and George Fullbright for technical assistance.

REFERENCES

1. Adham, I. M., Burkhardt, E., Benahmed, M., and Engel, W. (1993) *J. Biol. Chem. 268*, 26668−26672.
2. Adham, I. M., Burkhardt-Göttges, E., and Engel, W. (1996) *J. Anim. Breed. Genet. 113*, 229−235.
3. Tashima, L. S., Hieber, A. D., Greenwood, F. C., and Bryant Greenwood, G. D. (1995) *J. Clin. Endocrinol. Metab. 80*, 707−710.
4. Pusch, W., Balvers, M., and Ivell, R. (1996) *Endocrinology 137*, 3009−3013.
5. Zimmermann, S., Schottler, P., Engel, W., and Adham, I. M. (1997) *Mol. Reprod. Dev. 47*, 30−38.
6. Büllesbach, E. E., and Schwabe, C. (1995) *J. Biol. Chem. 370*, 16011−16015.
7. Yang, S., Rembiesa, B., Büllesbach, E. E., and Schwabe, C. (1992) *Endocrinology 130*, 179−185.
8. Büllesbach, E. E., Yang, S., and Schwabe, C. (1992) *J. Biol. Chem. 267*, 22957−22960.
9. King, D. S., Fields, C. G., and Fields, G. B. (1990) *Int. J. Pept. Protein Res. 36*, 255−266.
10. Maruyama, K., Nagata, K., Tanaka, M., Nagasawa, H., Isogai, A., Ishizaki, H., and Suzuki, A. (1992) *J. Protein Chem. 11*, 1−12.
11. Sieber, P., Kamber, B., Hartmann, A., Jöhl, A., Riniker, B., and Rittel, W. (1977) *Helv. Chim. Acta 60*, 27−37.
12. Beyerman, H. C., Izeboud, E., Kranenburg, P., and Voskamp, D. (1979) Synthesis of methionine-containing peptides via their sulfoxides, in *Sixth American Peptide Symposium*, pp 333−336, Pierce Chemical Co., Rockford, Il.
13. Büllesbach, E. E., and Schwabe, C. (1991) *J. Biol. Chem. 266*, 10754−10761.
14. Adler, A. J., Greenfield, N. J., and Fasman, G. D. (1973) *Methods Enzymol. 27*, 675−735.
15. De Lean, A., Munson, P. J., and Rodbard, D. (1978) *Am. J. Physiol. 235*, E97−E102.
16. Kamber, B., Hartmann, A., Eisler, K., Riniker, B., Rink, H., Sieber, P., and Rittel, W. (1980) *Helv. Chim. Acta 63*, 899−915.
17. Spande, T. F., and Witkop, B. (1967) *Methods Enzymol. 11*, 498−506.
18. Burkhardt, E., Adham, I. M., Hobohm, U., Murphy, D., Sander, C., and Engel, W. (1994) *Hum. Genet. 94*, 91−94.
19. Baker, E. N., Blundell, T. L., Cutfield, J. F., Cutfield, S. M., Dodson, E. J., Dodson, G. G., Crowfoot Hodgkin, D. M.,

Hubbard, R. E., Isaacs, N. W., Reynolds, C. D., Sakabe, K., Sakabe, N., and Vijayan, N. M. (1988) *Philos. Trans. R. Soc. London B 319*, 369−456.
20. Eigenbrot, C., Randal, M., Quan, C., Burnier, J., O'Connell, L., Rinderknecht, E., and Kossiakoff, A. A. (1991) *J. Mol. Biol. 221*, 15−21.
21. Creighton, T. E. (1984) in *Proteins: structures and molecular properties*, W. H. Freeman and Co., New York.

BI982687U