Chemical Synthesis of a Zwitterhormon, Insulaxin, and of a Relaxin-like Bombyxin Derivative†

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ABSTRACT: The structural motif of insulin and relaxin is frequently seen in molecules of divergent functions and origins. The insect developmental factor bombyxin, the relaxin-like factor from Leydig cells, and the insulin-like factor 4 (INSL4) all are made of two disulfide-linked chains and have one disulfide bond within the A-chain. The polyclonal antibody R6, which was raised against porcine relaxin, reacts with a wide variety of naturally occurring relaxins from primates, marine and terrestrial mammals, and elasmobranchs but does not recognize insulin. The antibody binds mainly to the arginines that occur in the *N*, *N*+4 positions in the B-chains of all relaxins which also constitute the receptor-binding site. The receptor-binding haptens were incorporated by total synthesis into human despentapeptide insulin and bombyxin II, a developmental factor from the silk moth *Bombyx mori*. In the process the insect factor became a perfect antigen for the anti-relaxin antibody, whereas the human insulin was transformed into a *bona fide* relaxin. The conversion was affected by changing four critical residues so that the insulin activity was retained to the extent of 10% of the original level. This, to the best of our knowledge, is the first designer protein to incorporate two unrelated biological functions in one primary sequence, and we are therefore proposing that, analogous to zwitterion, the generic name "Zwitterhormon" (German spelling) be used for this type of construct.

Relaxin is a proteohormone of insulin-like structure (Schwabe & McDonald, 1977; Schwabe et al., 1976, 1977). According to X-ray crystallographic analysis, insulin and relaxin have similar three-dimensional structures (Eigenbrot et al., 1991) but no biological and immunological cross-reactivities (Rawitch et al., 1980). Naturally occurring relaxins from different species show high sequence variability, but all trigger qualitatively the same biological response on the reproductive tract in mice or guinea pigs (Schwabe & Büllesbach, 1994). Besides the structurally important cysteines, there are only five residues absolutely constant, i.e., the glycines in positions B12 and B24, a glycine in position A14, and two arginine residues in positions B13 and B17 (the numbering system is based on human relaxin II). Although three of these residues are important for relaxin activity, only the arginines (B13, B17) are essential for receptor contact (Büllesbach et al., 1992; Rembiesa et al., 1993). The A14 position uniquely and critically controls the conformation of the whole molecule (Büllesbach & Schwabe, 1994).

Bombyxin is an insect developmental factor that can be isolated from the brain of the silk moth *Bombyx mori*. The primary structure (Nagasawa et al., 1986) as well as the three-dimensional structure as determined by NMR spectroscopy (Nagata et al., 1995) suggest that bombyxin has an insulin-like structure. The silk moth contains a family of these molecules of which only bombyxin II has the two active site arginines corresponding to the receptor-binding site of relaxin.

In spite of the high sequence variability of relaxins from different species the polyclonal rabbit anti-porcine relaxin antibody R6 could be used to elucidate the physiological significance of relaxin in tissues and body fluids of different species (O'Byrne et al., 1978; Steinetz et al., 1987, 1988, 1992). This is in harmony with our proposal that R6 binds to the only common motif in relaxins, the receptor-binding site. In this paper we are reporting the construction of a Zwitterhormon, insulaxin from insulin, and a zwitterligand for the anti-relaxin antibody R6 from the invertebrate developmental factor bombyxin II.

EXPERIMENTAL PROCEDURES

Materials

Chemicals. Derivatized amino acids for peptide synthesis were purchased from Bachem California (Torrance, CA) and Bachem Bioscience (Philadelphia, PA). High purity solvents from Burdick and Jackson (Muscataway, MI) were used for peptide synthesis and high-performance liquid chromatography. Reagents for automatic peptide synthesis were obtained from Perkin Elmer Applied Biosystems.

Proteins. Porcine relaxin was isolated from ovaries of pregnant sows as described (Büllesbach & Schwabe, 1985). For the present studies B29-porcine relaxin was used [B29-relaxin has a B-chain of 29 amino acid residues ending in Arg(B29)]. Porcine insulin was a gift of Eli Lilly. All other relaxins and analogs thereof were chemically synthesized,

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using a combination of solid-phase peptide synthesis and site-directed disulfide bond formation as previously described (Büllesbach & Schwabe, 1991, 1993, 1994, 1995; Büllesbach et al., 1992, 1994; Rembiesa et al., 1993). The syntheses of bombyxin II and analogs of insulin and bombyxin are described below.

Methods

High-Performance Liquid Chromatography (HPLC).[1] The mobile phase in all HPLC systems consisted of 0.1% trifluoroacetic acid (TFA) in water for solvent A and 0.1% TFA in 80% acetonitrile for solvent B. Gradients are expressed as %B and are given with the corresponding protocols.

Semipreparative HPLC was performed on a Waters HPLC system (Waters, Milford, MA) consisting of two pumps (model 6000A), a gradient programmer (model 680), a Rheodyne injector equipped with a 2 mL sample loop, a Uvicord S monitor (LKB, Bromma, Sweden) equipped with a 226 nm filter, and a Synchropak RP-P column (Synchrom Inc. Linden, IN) (C18, 10 mm × 250 mm). Approximately 5−20 mg of peptide was injected eventually in multiple steps, and elution was achieved at a flow rate of 3 mL/min. Peptide containing eluate was manually collected and lyophilized.

Analytical HPLC system 1 consisted of a two-pump Waters HPLC system, a UV monitor (Waters, Lambdamax model 481) set to 230 nm, and an automatic sample injector. For gradient programming and data collection, a Digital computer (Professional 380, Digital Equipment Corporation, Maynard, MA) was employed. As stationary phase a Synchropak RP-P column (C18, 4.1 mm × 250 mm) was chosen and guarded by a precolumn of Co:Pell ODS (Whatman, Clifton, NJ). Separations were achieved by linear gradients of 25−45% for bombyxin and 30−50% for insulin and relaxin over 30 min at a flow rate of 1 mL/min.

Analytical HPLC system 2: An Aquapore 300 C_8 column (2.1 mm × 30 mm) (Applied Biosystems, Foster City CA) was used in combination with an ABI-chromatograph (model 130A). Separations were performed at a flow rate of 100 μL/min. Samples of about 1 μg were injected by means of a Rheodyne valve equipped with a 50 μL sample loop, and the effluent was recorded at either 230 or 215 nm.

Amino Acid Composition. Peptides were hydrolyzed in vapor phase in 6 N HCl in the presence of 1% of phenol at 110 °C for 24 h. Thereafter the amino acids were derivatized with phenylisothiocyanate and analyzed using the Waters' Picotag system. Under the described conditions the hydrolyses of the Ile−Val bonds in insulin and bombyxin and the Val−Ile bond in relaxin are incomplete. Extended hydrolyses up to 72 h resulted in theoretical amounts for Ile and Val. Cysteine was detected but not quantified, and tryptophan was determined by UV spectroscopy.

Protein Determinations. Protein determinations were based on UV absorbance on an Olis Cary-15 spectrophotometer conversion (On-Line Instrument Systems, Inc.). Calculation of absorbance coefficients at 280 nm was based on tyrosine and tryptophan content of the molecules [1340 L cm^{-1} M^{-1} for tyrosine and 5600 L cm^{-1} M^{-1} for tryptophan (Long, 1968)].

Peptide Synthesis: Insulin Analogs. Insulin A-chains were synthesized by solid-phase peptide synthesis using Fastmoc chemistry on an ABI-430A automatic peptide synthesizer (Perkin Elmer Applied Biosystems) starting with 0.25 mmol of Fmoc-Asn(Trt) loaded onto hydroxybenzyloxybenzyl resin. The recommended trifluoroacetic acid-labile side chain-protecting groups were used for all trifunctional amino acids except the sulfhydryl groups in Cys(A7) and Cys(A20) which were acetamidomethyl (Acm) and methylbenzyl (MBzl) protected, respectively. Deprotection of the peptidyl resin was performed with trifluoroacetic acid in the presence of 9% (v/v) thiophenol (50 mg of peptidyl resin/ml) for 1.5 h at room temperature, the resin was filtered off, the peptide was precipitated with ether, and the pellet was collected by centrifugation. The A-chain was suspended in 50 mM NH_4HCO_3, the pH adjusted to about 9.5−10 with NH_3, and the peptide desalted on Sephadex G25 in 50 mM NH_4HCO_3. The peptide-containing fractions were pooled. In order to facilitate oxidation of the intrachain disulfide bond, the solution was supplemented with 25−50% (by volume) of dimethyl sulfoxide (Tam et al., 1991). Progress of the oxidation was recorded by Ellman reaction (Ellman, 1959), and usually oxidation was completed within 24 h at room temperature. The insulin A-chain was desalted on Sephadex G25 in 50 mM NH_4HCO_3 and lyophilized. Further purification was achieved by ion exchange chromatography on DEAE-cellulose at pH 8.5 in 25 mM Tris-HCl in the presence of 7 M urea employing a linear gradient from 0 to 0.3 M NaCl (300 mL each). After desalting on Sephadex G25 in 50 mM NH_4HCO_3 and lyophilization, the A-chain was suspended in about 5−10 mL of water, ammonia was added to dissolve the A-chain, and remaining salt was finally removed by dialysis against water (MW cutoff 3500). The yield after lyophilization was 159 mg (24.8% based on the first amino acid on the resin).

Full-length insulin B-chains were synthesized on Pam-resin preloaded with Boc-Ala and insulin-despentapeptide amide B-chains on methylbenzhydrylamine resin. Boc-chemistry was employed in combination with the ABI-430A automatic peptide synthesizer. HF-labile side chain-protecting groups were used for all trifunctional amino acids except the two cysteines which were protected by the HF-stable acetamidomethyl group in position B7 and the thiol-protecting and activating *S*-2-(3-nitropyridinesulfenyl) group in position B19. Cysteine-protected insulin B-chains were obtained after HF deprotection with 5% *m*-cresol as scavenger and HPLC purification. The yield was 31% based on the deprotected crude peptide.

The combination of A- and B-chains is demonstrated on the synthesis of insulaxin. Prior to chain combination the methylbenzyl-protected cysteine of the A-chain (22 mg, 8.8 μmol) was liberated with 2 mL of HF in the presence of 5% *m*-cresol for 45 min at 0 °C. The HF was removed in a stream of nitrogen and the peptide precipitated with ether

[1] Abbreviations: Acm, acetamidomethyl; BSA, bovine serum albumin; G-bombyxin, Pro(A10)→Gly bombyxin; GLEV-bombyxin, Pro(A10)→Gly, Tyr(B9)→Leu, His(B13)→Glu, Ala(B15)→Val bombyxin; GRER-insulin, Ile(A10)→Gly, Ser(B9)→Arg, His(B10)→Glu, Glu(B13)→Arg insulin; GRER-dpp-insulin, Ile(A10)→Gly, Ser(B9)→Arg, His(B10)→Glu, Glu(B13)→Arg insulin despentapeptide (B26−30) amide; HEPES, *N*-(2-hydroxyethyl)piperazin-*N*′-(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; LE-bombyxin, Tyr(B9)→Leu, His(B13)→Glu, bombyxin; PMSF, phenylmethanesulfonylfluoride; RIA, radioimmunoassay; RER-insulin, Ser(B9)→Arg, His(B10)→Glu, Glu(B13)→Arg insulin; RR-insulin, Ser(B9)→Arg, Glu(B13)→Arg insulin; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

and collected by low-speed centrifugation. After drying over KOH (30 min), the A-chain was dissolved in 4 mL of 8 M guanidinium chloride containing 0.1 M acetic acid/NaOH, pH 4.5. This solution was added to equimolar amounts of B-chain, and the reaction was stirred for 24 h at 37 °C and separated on Sephadex G50 sf in 1 M acetic acid. The protein-containing fractions were lyophilized and further purified by preparative HPLC. The second interchain disulfide link was synthesized with iodine in 70% acetic acid (Sieber et al., 1977) at a protein concentration of 1 mg/mL and a 50-fold excess of iodine. The reaction was quenched with ascorbic acid and desalted on Sephadex G25 in 1 M acetic acid, lyophilized, and further purified by preparative HPLC using a linear gradient from 30 to 50% B over 30 min. Yield: 7.45 mg = 16.1%.

Bombyxin-II and Bombyxin Analogs. The bombyxin A-chain was synthesized via Fmoc chemistry on hydroxybenzyloxybenzyl resin. All side chain-protecting groups were TFA-labile except the protection in cysteine (A7) (acetamidomethyl, Acm) and cysteine A20 (4-methylbenzyl, MeBzl). Syntheses were performed either on a semiautomatic peptide synthesizer (Vega Coupler 1000) or by automatic peptide synthesis (ABI-430A). On the semiautomatic synthesizer, condensation was performed with dicyclohexylcarbodiimide/1-hydroxybenzotriazole (DCC/HOBt) for 3 h and repeated when incomplete coupling was detected by the ninhydrin test (Kaiser, 1970), and in automatic peptide synthesis 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was utilized in Fastmoc chemistry. Condensation of arginine in position A9 as Fmoc-Arg(Pmc)OH was very slow and remained incomplete even after extended condensation times and recoupling, i.e., in semiautomatic synthesis the first condensation of Arg was performed for 6 h, followed by a second condensation for 8.5 h and capping with acetic anhydride. During automatic synthesis, the first condensation was 66 min followed by a recoupling of 4.7 h. In any case the incorporation of arginine was about 70% as determined by amino acid composition. Deprotection, formation of the intrachain disulfide bond, and purification was performed as described for the insulin A-chain. Ion exchange chromatography was sufficient to remove truncated or arginine deficient bombyxin A-chain. Starting with 0.25 mmol of initial amino acid, 126 mg of purified bombyxin A-chain was obtained. Amino acid composition: Asp 2.16 (2), Ser 2.00 (2), Glu 1.04 (1), Pro 1.05 (1), Gly 1.03 (1), Val* 2.71 (3), Ile* 0.56 (1), Leu 3.05 (3), Tyr 0.94 (1), Arg 0.99 (1) [*low values due to incomplete hydrolysis of the Ile−Val (A2, A3) bond].

Bombyxin B-chain was synthesized using Boc-chemistry on an ABI 430A peptide synthesizer similar to the synthesis of the insulin B-chain except that tryptophan was N^{in}-formyl-protected. The peptidyl resin was HF-deprotected with *m*-cresol as scavenger (5 vol %). The peptide was extracted into 1 M acetic acid, lyophilized (yield: 874 mg), and purified by semipreparative HPLC, using a linear gradient from 30% to 52% B over 30 min (yield 191 mg, 21.9%). Amino acid composition: Asp 2.01 (2), Thr 1.91 (2), Glu 3.72 (4), Pro 1.05 (1), Gly 2.32 (2), Ala, 3.95 (4), Val 1.89 (2), Leu 2.85 (3), Tyr 0.88 (1), His 1.93 (2), Arg 1.98 (2).

The combination of the bombyxin chains was performed as described for the combination of the insulin chains starting with 34 mg of bombyxin A-chain. HPLC-purified bombyxin derivative containing two Acm groups in positions A7 and B10 and N^{in}-formyl in Trp(B23) was obtained in a yield of 28.9% (20.9 mg). After formation of the third disulfide, bombyxin containing an N^{in}-formyl group in B23 was obtained in an overall yield of 12.5 mg (17.9%). The tryptophan side chain was liberated by treatment with 2 mL of 10% aqueous piperidine for 2 min at room temperature, and the reaction was acidified with 0.2 mL of glacial acetic acid and separated by HPLC. After lyophilization 10.0 mg of bombyxin was obtained (overall yield: 13.9%).

Biological Assays: Relaxin Tracers. Tracers were generated by two different procedures: (a) formyltyrosyl porcine relaxin was iodinated by the chloramine T method, and labeled relaxin was separated by gel filtration on Sephadex G25 in 50 mM phosphate buffer (Schwabe, 1983); (b) ([^{125}I]3,5-diiododesaminotyrosyl(A7))porcine relaxin and [^{125}I]3,5-diiododesaminotyrosyl(A1))porcine relaxin were generated by reacting pre-iodinated Bolton−Hunter reagent with porcine relaxin at pH 7.5, followed by HPLC separation of the four isomers (Yang et al., 1992).

Insulin Tracer. ^{125}I-Tyr(A14)porcine insulin was prepared as described by Linde et al. (1986).

Radioimmunoassays. (RIA) were performed in 50 mM phosphate buffer, pH 7.4, and 150 mM NaCl supplemented with 1% BSA and 0.01% NaN_3. Sample (100 μL), tracer (100 μL, 15 000−20 000 cpm), and R6 antibody serum (diluted 1:30 000, 100 μL) were added in succession and were incubated for 8−16 h at 4 °C. Thereafter, bound and free tracer was separated with goat anti-rabbit γ-globulins (Antibodies Incorporated, Davis, CA), covalently linked to CNBr-activated cellulose. Immunosorbant was added and the reaction shaken for 1 h at room temperature. Wash solution (3 mL of 50 mM phosphate buffer supplemented with 0.1% Tween 20) was added, and the cellulose beads were centrifuged off in an IEC Centra-7 centrifuge (International Equipment Company, Needham Hts, Mass) at 3000 rpm for 5 min. The supernatant was discarded and the pellet was washed twice with 3 mL of wash buffer and thereafter counted in a γ-counter (LKB Minigamma, Bromma Sweden). A slightly different assay was used in the laboratory of Dr. Steinetz (O'Byrne & Steinetz, 1976).

Receptor-Binding Assays. Receptor-binding assays on crude membrane preparations of mouse tissue were performed as described before (Yang et al., 1992). Two whole mouse brains were dissected and dropped into 15 mL of chilled buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 0.2 mM PMSF, and 80 mg/L soybean trypsin inhibitor, pH 7.5) supplemented with sucrose (0.25 M), and homogenized three times for 10 s with a Polytron homogenizer (Brinkmann, Westbury, NY) at setting 5. The homogenate was centrifuged at 700*g* for 10 min at 4 °C, and the pellet was again homogenized in 15 mL of the sucrose-containing buffer and centrifuged under the same conditions. The pellet was discarded, and the supernatants were combined and centrifuged for 1 h at 20000*g*. The resulting pellet was suspended in buffer without sucrose addition and centrifuged again for 1 h at 20000*g*.

For binding assays, crude membranes of two mouse brains were suspended in 1 mL of binding buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 0.2 mM PMSF, 1% BSA, 2.8 mM glucose, 1.6 mM $CaCl_2$, 0.025 mM $MgCl_2$, and 1.5 mM $MnCl_2$). Aliquots of 40 μL of crude membranes were added to 40 μL of [^{125}I]diiododesaminotyrosyl(A1)-porcine relaxin

Table 1

relaxin derivative	relaxin activity: bioactivity[a]	relaxin receptor-binding[b] ED_{50}	antibody R6 binding
human relaxin II	+++	1−6 ng/mL	100.0%
Human Relaxin with Modifications in the A-Chain			
Ala(A14) human relaxin II	++	10−15 ng/mL	89.0%
Ile(A14) human relaxin II	+	150 ng/mL	59.0%
Human Relaxin with Modifications in the B-Chain			
Cit(B13),Cit(B17) human relaxin II	−	3800 ng/mL	<13.0%
Cit(B13) human relaxin II	−	6500 ng/mL	<13.0%
Cit(B17) human relaxin II	−	3500 ng/mL	<13.0%
Lys(B13),Lys(B17) human relaxin II	−	4500 ng/mL	<13.0%
Ala(B17) human relaxin II	−	4500 ng/mL	<13.0%
Gln(B14) human relaxin II	++	1−6 ng/mL	76.0%
Asp(B14) human relaxin II	+++	1 ng/mL	0.5%

[a] Mouse symphysis pubis assay: (+++) fully active; (++) partially active at 1 μg/mouse; (+) partially active at 5 μg/mouse; (−) inactive at 20 μg/mouse. [b] Receptor-binding assays on crude membranes of mouse brain < inactive at the highest concentration measured.

(100 000 cpm) and 20 μL of buffer (total binding) or 20 μL of the corresponding relaxin analog (dose response) in 1.5 mL Eppendorf vials. The assay was incubated for 1 h at room temperature, and the reaction was diluted with 1 mL of ice-cold wash buffer (25 mM HEPES, 0.14 M NaCl, 5.7 mM KCl, 1% BSA, 0.01% NaN_3) and centrifuged for 10 min at 14 000 rpm at room temperature. Thereafter, the supernatant was discarded and the tip of the vial cut and counted in a gamma counter (Minigamma, LKB, Sweden). Remaining radioactivity at the highest relaxin concentration was considered nonspecific binding. Total binding was usually about 7−10% of the total counts added, and specific binding was about 30−50% of the total binding.

Receptor-Binding Assay on Crude Membrane Preparations of Rat Tissues. Sprague Dawley rats (female 150−180 g) were primed with 5 μg of β-estradiol 17-cypionate in 100 μL of sesame oil. Five days later the rats were killed in an atmosphere of CO_2 and brain and uterus dissected. Crude membranes were prepared as described for mouse tissue. The membranes prepared from one animal were sufficient to generate one dose−response curve with similar properties for relaxin standard as described above for mouse tissue.

Receptor-Binding Assays on Crude Membrane Preparations of Human Placenta. Insulin receptor-binding assays were performed on crude membrane preparations of human term placenta. Crude membranes were prepared as described in the literature (Hock & Hollenberg, 1980). Assays were performed in 1.5 mL Eppendorf tubes at room temperature for 60 min in a total volume of 0.1 mL of binding buffer (25 mM HEPES, pH 7.4, containing 5 mM $MgCl_2$ and 104 mM NaCl supplemented with 0.2% BSA, 0.01% soybean trypsin inhibitor, and 1 mM PMSF). Dose−response dependence was established by using 50 000 cpm of ^{125}I-Tyr(A14)-insulin and various concentrations of unlabeled ligand. Binding was terminated by addition of 1 mL of chilled binding buffer and bound and free tracer separated by centrifugation in a microcentrifuge (10 min at 14 000 rpm). The supernatant was discarded, and the tip of the vial was cut and counted in a gamma counter (Minigamma, LKB, Sweden). Remaining radioactivity at insulin concentrations of 2 μg/mL was considered nonspecific binding. Total binding was usually about 40% of the total counts added, and specific binding was about 90% of the total binding.

Mouse Symphysis Pubis Assay. was performed as described (Steinetz et al., 1960). Ovariectomized virgin female mice were primed with 5 μg of β-estradiol 17-cypionate in 100 μL of sesame oil. Five days later groups of five mice were injected with various doses of relaxin dissolved in 1% benzopurpurin 4B. For positive control porcine or human relaxin (1 μg/mouse) and for negative control 1% benzopurpurin 4B solution in water were used. After 16 h the mice were killed in an atmosphere of CO_2, the symphysis pubis was dissected free, and the distance between the interpubic bones was measured under a dissecting microscope fitted with transilluminating fiberoptics. Usually, three independent assays were performed for each derivative.

RESULTS AND DISCUSSION

The structural requirements for relaxin-binding have been examined using a wide variety of relaxins and relaxin derivatives as well as other hormones such as insulin and bombyxin-II. The ability of the derivatives to elicit a response were compared in the intact mouse (Steinetz et al., 1960) by binding to the relaxin receptor crude membrane preparations of rat and mouse brain and uteri (Yang et al., 1992) and by cross-reactivity to the R6 antibody. Either native porcine relaxin or human relaxin II was used as standard (Table 1).

These studies have been greatly facilitated by our synthetic approach which allows one to produce the insulin-like disulfide pattern unambiguously by selective deprotection and activation (Büllesbach & Schwabe, 1991). Successful syntheses of relaxins of different species (Büllesbach & Schwabe, 1991, 1993; Büllesbach et al., 1994), insulin (Akaji et al., 1993; Büllesbach & Schwabe, 1994), and human Leydig cell-derived relaxin-like factor (RLF) (Büllesbach & Schwabe, 1995) have been reported. The method appears to work without fail, whereas the random chain combination can be problematic (Chu et al., 1987, 1994; Gattner et al., 1981). In fact, the production of Arg(B9)Arg(B13)-insulin[2] was attempted either by combining synthetic B-chain and native A-chain (Gattner et al., 1981; Katsoyannis & Tometsko, 1966) or by the directed disulfide synthesis. While no product was obtained from the random chain combination, the sequential disulfide bond formation resulted in a product

[2] The numbering system for relaxins and bombyxins is based on the sequence of human relaxin II. For all insulin analogs, the insulin numbering system is used.

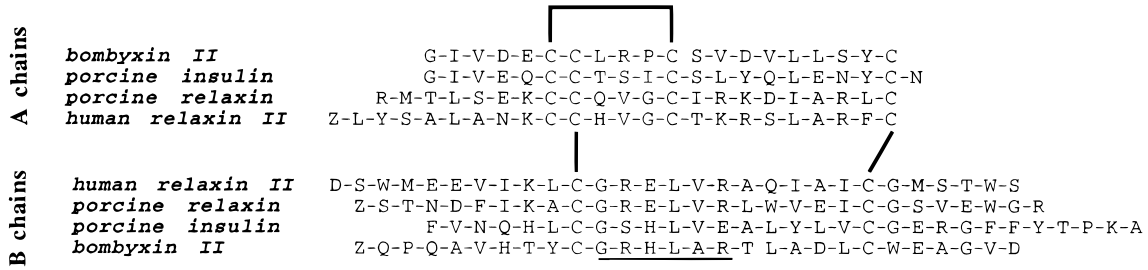


FIGURE 1: Sequence comparison of human relaxin II, porcine relaxin, bombyxin II, and porcine insulin (Z = L-pyroglutamic acid). The underlined sequence corresponds to the active site of relaxin.

comparable in yield to other insulin analogs produced by this procedure (Büllesbach & Schwabe, 1994). The invertebrate developmental factor bombyxin II was synthesized by the methods developed for relaxin. A slightly different synthesis for bombyxin has been published by Maruyama et al. (1992).

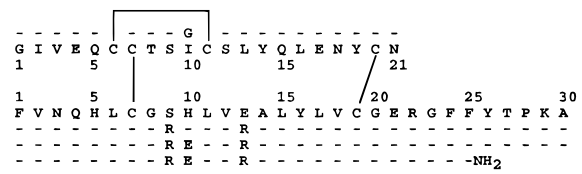
All analogs were HPLC-purified, and their chemical integrity was confirmed by reversed-phase HPLC of the intact and the reduced molecules, by amino acid composition, and, for selected analogs, by mass spectrometry.

Human relaxin II and porcine relaxin differ by 57% in their primary sequence (Figure 1), yet they are equipotent in the mouse bioassay (Büllesbach & Schwabe, 1991; Ferraiolo et al., 1989) and receptor-binding assays (Büllesbach et al., 1992; Yang et al., 1992). In the radioimmunoassay with the R6 antibody and ^{125}I-labeled porcine relaxin, synthetic human relaxin II showed an effective dose (ED_{50}) of 1 nM compared to 0.2 nM for porcine relaxin. We choose to use human relaxin as the reference molecule.

The receptor-binding site of relaxin involves two B-chain arginines, separated by one pitch of the α-helix (Büllesbach et al., 1992). When these residues were modified, relaxin activity ceased in parallel with R6 antibody interaction (Table 1). The same modifications did not inhibit binding to other anti-relaxin antibodies, and this observation confirms that R6 is directed toward the receptor-binding region of relaxin. While the relative potency and antibody (R6) binding propensity are qualitatively but not quantitatively parallel, it is none-the-less true that molecules, devoid of one or both of the receptor-binding arginines, do not bind the R6 antibody.

These results further support the proposition that the R6 antibody recognizes the receptor-binding site of relaxin, but that steric factors or neighboring residues in the molecules affect both R6 and receptor-binding differently. Changing Gly(A14), a conformational determinant in human relaxin, to isoleucine significantly reduces receptor-binding (Büllesbach & Schwabe, 1994) but to a lesser extent R6 binding. Other residues on the B-chain helix probably modulate receptor and R6-antibody-binding in different ways. Replacement of Glu(B14) by the uncharged glutamine has little effect on biological potency and none on the R6 affinity, whereas the introduction of the shorter aspartic acid suppresses the affinity of relaxin for the antibody significantly without measurable reduction in receptor binding. In summary, the different effects indicate that the same region is important for binding to both the receptor and the antibody, but that the conformation for receptor binding differs in a subtle way from the conformation for R6 binding.

Introducing the purported relaxin receptor-binding site into insulin by total synthesis at a position equivalent to where it



Insulin analog	R6 binding [%]
human relaxin	100.0
R(B9)R(B13) insulin	25.0
G(A10)R(B9)R(B13) insulin	33.0
R(B9)E(B10)R(B13) insulin	86.0
G(A10)R(B9)E(B10)R(B13) insulin	98.0
G(A10)R(B9)E(B10)R(B13) insulin despentapeptide amide	420.0

FIGURE 2: Primary structures of porcine insulin and analogs and their relative affinity to anti-porcine relaxin antibody R6 (human relaxin II = 100%).

is found in relaxin provided proof for the identity of the antigenic site and further evidence that, after the A-chain loop correction had been made, relaxin and insulin attained the same conformation (Figure 2). The replacement of serine(B9) and glutamic acid(B13) in porcine insulin by arginine (RR-insulin) improved the binding of insulin to the anti-relaxin antibody to 25% compared to human relaxin (100%). Further remarkable improvement of antibody binding up to 86% was achieved when His(B10) was replaced by glutamic acid (RER-insulin). Finally, the change of the large amino acid Ile(A10), which typifies insulin, to the relaxin-specific glycine (GRER-insulin) made the hybrid indistinguishable from human relaxin in the RIA. It came as a surprise that removal of the C terminus of the B-chain of GRER-insulin to the despentapeptide (GRER-dpp) insulin-amide caused a 420% increase in R6 binding compared to human relaxin or 100% compared to porcine relaxin, the original antigen (Figure 3).

Receptor binding is a crucial test which was performed on three different tissues, i.e., membranes of human placenta and, due to the absence of relaxin receptors in the placenta membranes, rat uterus (Osheroff et al., 1990) and brain (Osheroff & Phillips, 1991). The results (Figure 4) show clearly that the changes in insulin have resulted in a molecule that binds the relaxin receptor competitively, and Figure 5 shows that insulin's typical properties have been retained in our construct (GRER-dpp insulin). The conclusion seems justified that a four amino acid exchange in insulin has given us a molecule with R6 binding properties identical to those of human relaxin II and, most importantly, a *bona fide* relaxin.

Although this work is in its early stages, some interesting conclusions may be drawn. The reader familiar with

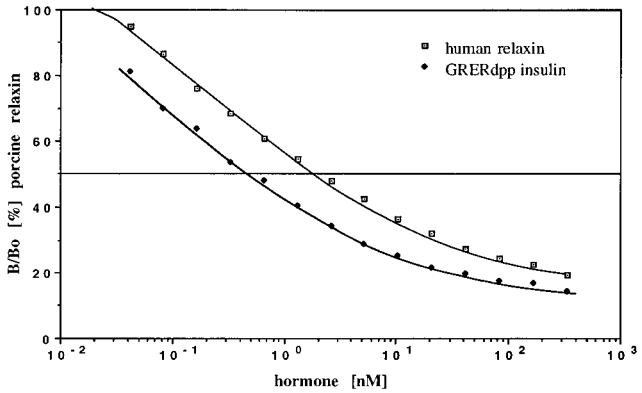


FIGURE 3: RIA of GRER-dpp insulin compared to human relaxin II as standard. The assay was performed with anti-porcine relaxin antibody R6 and ([^{125}I]3,5-diiododesaminotyrosyl(A7))porcine relaxin for tracer.

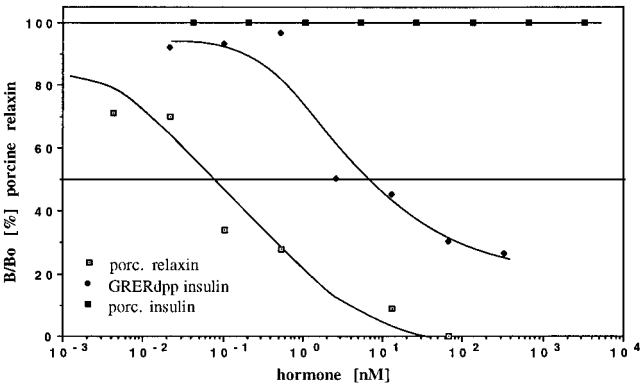


FIGURE 4: Receptor-binding assay of GRER-dpp insulin, insulin, and porcine relaxin to crude membrane preparations of rat brain. ([^{125}I]3,5-Diiododesaminotyrosyl(A1))porcine relaxin was used for tracer.

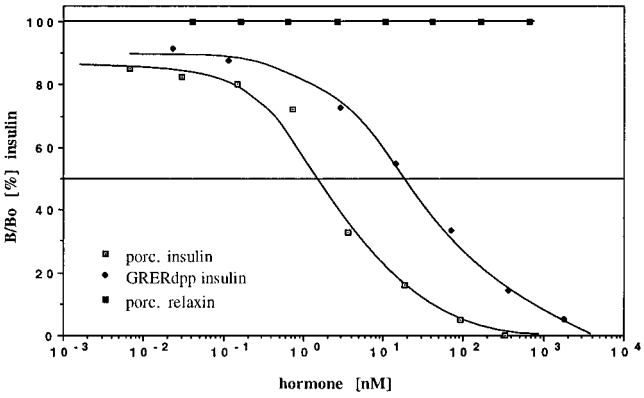


FIGURE 5: Receptor-binding assay of GRER-dpp insulin, insulin, and porcine relaxin to crude membrane preparations of human placenta using [^{125}I-Tyr(A14)] porcine insulin for tracer.

probability calculations may note that the random mutational change of insulin to a relaxin could involve as many as 20 $\times$ 10^{50} trials! Held against the four trials actually needed, this number constitutes a numerical expression of the advantage of scientific methods.

Another startling conclusion is that structures that differ by about 75% in their primary sequence can have nearly the same conformation, and finally one could marvel at the fact that glycine, as far away from the binding site as Gly(A14) is in human relaxin, can assert such a relatively long distance effect. Certainly this effect will be noticed by those who aspire to protein design by first principle.

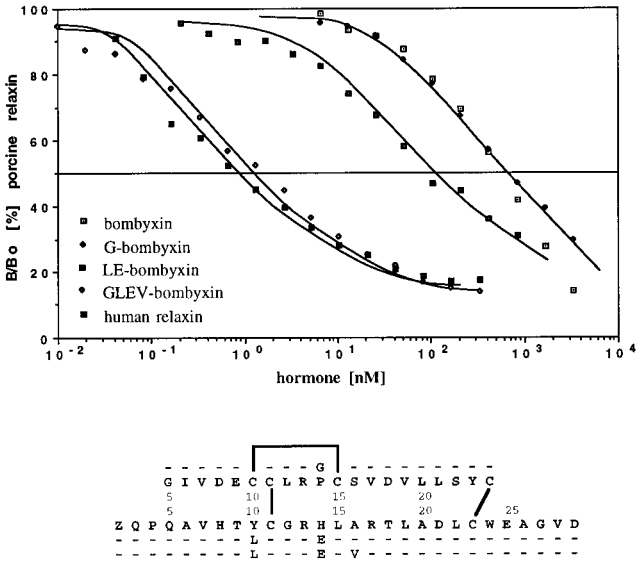


FIGURE 6: RIA of bombyxin II and its analogs in comparison with human relaxin II (upper panel). Primary structures of bombyxin II and analogs (lower panel). ([^{125}I]3,5-diiododesaminotyrosyl(A7))-porcine relaxin was used for tracer.

Bombyxin II, a developmental factor derived from the silk moth *B. mori* (Nagasawa, 1992), has a relaxin-like structure (Nagata et al., 1995), including the two arginines in the B-chain helix, but the cross-reactivity to the R6 antibody is only 0.1% compared to human relaxin (Figure 6). In light of the fact that significant cross-reactivity of RR-insulin to R6 has been observed, this result was rather unexpected. No improvement of cross-reactivity was obtained by replacing Pro(A14) with the Gly observed in relaxin (G-bombyxin). The replacement of Tyr(B10) by Leu, commonly seen in relaxin and His(B14) by Glu (LE-bombyxin), increased the affinity to the antibody only to 2.5% compared to human relaxin. With all the crucial amino acids identified so far, i.e., Gly in A14, Arg in positions B13, B17, and Glu in position B14, the effect on the R6 antibody affinity was surprisingly low. One obvious difference between the strong binding relaxin and insulin analogs and those of lesser affinity is a valine in position B18 which is alanine in bombyxin. The simultaneous change in bombyxin of Pro-(A14) to glycine, tyrosine(B10) to leucine, histidine(B14) to glutamic acid, and alanine(B18) to valine (GLEV-bombyxin) caused an increase in affinity to the R6 antibody to 63% compared to human relaxin. Thus, by modifying an insect hormone in four positions, we have gained substantial cross-reactivity to the polyclonal antibody R6. These experiments show quite clearly that the overall structures of the relaxin/insulin group of proteohormones are very similar in spite of high sequence variability. They also show that we are beginning to understand some of the structural subtleties that are important for receptor and antibody binding in small proteins.

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