Sauer, R. T., Pabo, C. O., Meyer, B. J., Ptashne, M., & Backman, K. C. (1979) *Nature (London) 279*, 396–400.
Weiss, M. A., Karplus, M., Pabo, C. O., & Sauer, R. T. (1986a) *Biochemistry* (submitted for publication).
Weiss, M. A., Karplus, M., & Sauer, R. T. (1986b) *Biochemistry* (submitted for publication).
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.

Preparation and Properties of Porcine Relaxin Derivatives Shortened at the Amino Terminus of the A Chain†

Erika E. Büllesbach* and Christian Schwabe
Department of Biochemistry, Medical University of South Carolina, Charleston, South Carolina 29425
Received April 2, 1986; Revised Manuscript Received June 18, 1986

ABSTRACT: Porcine relaxins shortened at the N terminus of the A chain were produced after protection of all amino groups with the base-labile [[(methylsulfonyl)ethyl]oxy]carbonyl (Msc) protecting group. The first two amino acids were removed by cyanogen bromide digestion whereby simultaneously a free α-amino group was generated in position A3. The resulting des-Arg^{A1},Met^{A2}-$N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-tris[[[(methylsulfonyl)ethyl]oxy]carbonyl]relaxin was further shortened by preparative Edman degradation. The shortest derivative obtained was des-Arg^{A1},Met^{A2},Thr^{A3},Leu^{A4},Ser^{A5},Glu^{A6}-$N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-tris[[[(methylsulfonyl)ethyl]oxy]carbonyl]relaxin. The deprotection of the derivatives in alkaline media resulted in crude des-A(1–2)- to des-A(1–6)-relaxins, which were subsequently purified by gel filtration on Sephadex G-50 superfine followed by either ion exchange chromatography on CM-cellulose at pH 5.1 or high-performance liquid chromatography on reversed-phase columns. During the CNBr digest, a side product was isolated that was identified as the corresponding homoserine ([Hse^{A2}]relaxin) derivative. Shortened relaxin derivatives and [Hse^{A2}]relaxin were characterized by reversed-phase chromatography, electrophoresis, end-group determination, and amino acid composition. Circular dichroism studies revealed a distinct change in the structure of relaxins that were shortened by three and more amino acid residues. In the mouse interpubic ligament assay, des-A(1–2)-relaxin and [Hse^{A2}]relaxin were fully biologically active while the bioactivity of des-A-(1–3)-relaxin dropped to about 50%. Relaxins shortened by four and more amino acid residues were biologically inactive. The N-terminal region of the A chain of porcine relaxin appears to be important for the structural integrity of the hormone.

Relaxin, a hormone of pregnancy, causes the widening of the mammalian birth canal during late pregnancy and inhibits uterine contractions. Like insulin, the relaxin molecule consists of two polypeptide chains linked by inter- and intrachain disulfide bonds. The positions of the disulfide links are similar to those in insulin (Schwabe & McDonald, 1977).

Naturally occurring relaxins differ in their primary structure and in their biological potency over a wide range. For instance rat relaxin (John et al., 1981; Hudson et al., 1981) and pig relaxin (Schwabe et al., 1976, 1977; James et al., 1977) have a similarity of only 54%. However, pig relaxin is more potent than rat relaxin (Sherwood, 1979) in the mouse interpubic ligament assay. Shark relaxins (Gowan et al., 1981; Büllesbach et al., submitted for publication) differ from pig relaxin by about 48–50% and show low activity in the symphysis pubis relaxation assay as well as in the uterine relaxation assay (Gowan et al., 1981; Büllesbach et al., submitted for publication). Comparison of the primary sequences of all known relaxins (John et al., 1981; Schwabe et al., 1976, 1977; James et al., 1977; Gowan et al., 1981; Haley et al., 1982; Hudson et al., 1981, 1983, 1984) reveals that there is no invariant amino acid residue in the N-terminal region of the relaxin A chain (Figure 1), and we therefore decided to investigate the relevance of this portion of the relaxin molecule by chemically modifying native porcine relaxin.

Porcine relaxin (B29) can be prepared in relatively large quantities free of relaxin molecules that vary in length at the N terminus of the A chain (Büllesbach & Schwabe 1985a,b) and at the C terminus of the B chain (Büllesbach & Schwabe, 1985b; Niall et al., 1980; Walsh & Niall, 1980) by a combination of preparative carboxypeptidase A digestion and chromatographic procedures. Pure B29 relaxin thus obtained consists of the 22-residue A chain and a 29-residue B chain (Büllesbach & Schwabe, 1985b). It has been known that N-terminal elongation of porcine relaxin by one amino acid does not affect the biological potency (Schwabe, 1983; Büllesbach & Schwabe, 1985a). Schwabe and Braddon (1976) described the partial loss of biological activity in des-Arg^{A1},Met^{A2}-relaxin (porcine); however, there remained some uncertainty as to whether the loss of bioactivity was caused by the absence of the first two amino acids or by other side reactions. Tregear et al. (1983) reported the total synthesis of porcine relaxin chains and their random combination and that the combination mixture of A(4–22) and B(1–25) was bioactive in the uterine motility suppression assay. The same procedure was used to produce a [Nle^{A2}]B28 porcine relaxin that was also active in the same assay system (Tregear et al., 1981). Symphysis pubis elongation assay results were not reported. In this paper, we describe the systematic shortening of porcine relaxin molecules at the N terminus of the A chain and of [Hse^{A2}]relaxin, as well as some physical and biological

† This work was supported by NIH Grant HD-10540 and NSF Grant PCM-8302194 and in part by a Medical University of South Carolina Biomedical Research Support Grant, 1984.
* Author to whom correspondence should be addressed.

0006-2960/86/0425-5998$01.50/0

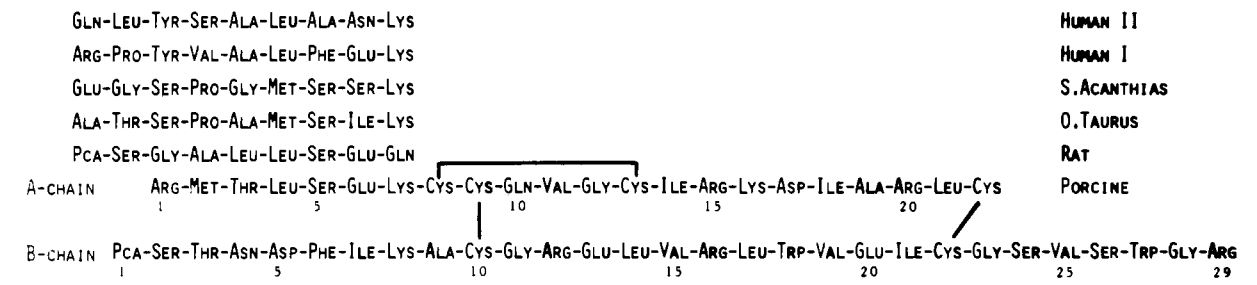

FIGURE 1: Primary structure of porcine relaxin and N-terminal sequences of A chains of relaxins from other species.

properties of these relaxin derivatives.

MATERIALS AND METHODS

Materials

Porcine relaxin was extracted from ovaries of pregnant sows, and the corresponding B29 derivative was prepared according to Büllesbach and Schwabe (1985b). Chemicals for chromatography were HPLC-grade. All other chemicals were analytically pure. Urea stock solution (7 M) was stored over a mixed-bed ion exchanger at 4 °C, and cyanogen bromide was sublimed before each experiment. Pyridine was distilled from ninhydrin, and phenyl isothiocyanate was twice distilled in vacuo under nitrogen and stored at 4 °C in the dark. [[(Methylsulfonyl)ethyl]oxy]carbonylsuccinimide ester (MscONSu) was synthesized according to Tesser and Balvert-Geers (1975).

Dialysis membrane tubing (Spectrapor 3, exclusion limit 3500) was purchased from American Scientific Products (McGaw Park, IL) and CM-cellulose (Whatman CM-52) from Reeve Angel (Clifton, NJ). Immature ICR mice (20–25 g) were used for bioassay.

Analytical Procedures

Cellulose–acetate electrophoreses were performed in a Deluxe electrophoresis chamber on cellulose–acetate strips (Sepraphore III, 2.5 cm × 17.1 cm), a product of Gelman Sciences, Ann Arbor, MI, at pH 4.8 (4 M urea, 0.1 M pyridine, acetic acid) for 1.5 h at 200 V and at pH 8.6 (4 M urea, 25 mM sodium barbital, 25 mM sodium acetate, 5 g/L ethylenediaminetetraacetate disodium salt, and 10 vol % dioxane) for 3 h at 200 V. The strips were stained in 0.2% Ponceau S in 3% trichloroacetic acid.

High-Performance Liquid Chromatography (HPLC). A Synchropak RP-P column (C_{18}) (4.1 mm × 250 mm) (Synchron Inc., Linden, IN), protected by a precolumn of Co:Pell ODS (30–38 μm) (Whatman, Clifton, NJ), was used for all analytical and preparative separations. The column eluent was pumped at 1.5 mL/min by a Waters HPLC system. The eluate was detected by a UV monitor at 226 nm (Uvicord S, LKB Product, Bromma, Sweden).

Amino acid analyses were performed on a Durrum D 502 analyzer (Dionex Instrument Co., Palo Alto, CA). Samples were hydrolyzed in vacuo in 6 N HCl at 120 °C for 24 h.

End group determinations were carried out by automatic Edman degradation in a Beckman 890 TC automatic sequencer using a 0.1 M quadrol program and with polybrene to aid retention of the relaxin in the sequencer cup. PTH-amino acids were identified by HPLC according to Schwabe et al. (1984).

UV spectra were obtained on a Cary 15 recording spectrophotometer (Cary Instruments, Atlanta, GA).

Circular dichroism measurements were performed on a Cary Model 60 CD spectrometer (Cary Instruments, Atlanta, GA) using a 0.05-cm path-length cuvette. Relaxin derivatives were dissolved to yield a concentration of 0.15–0.3 mg/mL in 0.05 M phosphate buffer, pH 7.0. The protein solutions were passed through Millipore filters (0.45 μm), and the protein content was determined by UV spectroscopy with specific absorption coefficients as outlined in Table II. The results are presented as mean residual ellipticities (Adler et al., 1973).

Preparative Procedures

Preparation of $N^{\alpha A1}$,$N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-Tetrakis[[[(methylsulfonyl)ethyl]oxy]carbonyl]B29 Relaxin (Msc_4-B29 Relaxin). Porcine relaxin (B29) (200 mg, 34.5 μmol) was suspended in 10 mL of dimethyl sulfoxide (Me_2SO) and 270 μL (1.94 mmol) of triethylamine, and 110 mg (415 μmol) of MscONSu dissolved in 10 mL of Me_2SO was added. The reaction was stirred at room temperature for 30 min whereafter a clear solution was obtained. The reaction was stopped by the addition of 500 μL of acetic acid and the product desalted on Sephadex G-25 medium in 1 M acetic acid. After lyophilization, 181 mg of Msc_4-B29 relaxin was obtained (81%).

Preparation of Des-Arg^{A1},Met^{A2}-B29 Relaxin. B29 relaxin (11.6 mg, 2 μmol) was dissolved in 200 μL of 70% trifluoroacetic acid. Cyanogen bromide (23 mg, 217 μmol) was added, and the reaction was incubated at room temperature overnight in the dark. The reaction mixture was diluted with water to about 5% trifluoroacetic acid and lyophilized. The residue was then dissolved in water, adjusted to pH 8, and dialyzed against 0.05 M NH_4HCO_3 for 10 h, followed by 10-h dialysis against water, and lyophilized. Purification was achieved by ion exchange chromatography on CM-cellulose at pH 5.1 (buffer: 7 M urea, 0.05 M NH_4OAc/AcOH, pH 5.1) (column: 1.5 cm × 20 cm). The relaxin derivatives were eluted with a linear NaCl gradient (0–0.2 M NaCl, 200 mL each), and the fractions obtained were dialyzed against deionized water (24 h) and lyophilized. Yields were as follows: des-Arg^{A1},Met^{A2}-B29 relaxin, 4.0 mg (36%); [Hse^{A2}]B29 relaxin, 2.6 mg (22%).

Preparation of $N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-Tris[[[(methylsulfonyl)ethyl]oxy]carbonyl]-des-Arg^{A1},Met^{A2}-B29 Relaxin (Msc_3-des-A(1–2)-B29 Relaxin). Msc_4-B29 relaxin (200 mg, 31.3 μmol) was dissolved in 3 mL of 70% trifluoroacetic acid and treated with 400 mg of CNBr as described for des-Arg,-Met-B29 relaxin. After 10 h, the mixture was diluted with water to a final concentration of 5% trifluoroacetic acid and lyophilized. The resulting crude product was dissolved in 6 M guanidinium chloride and desalted on Sephadex G-25 medium in 1 M acetic acid and lyophilized (yield: 168 mg, 90%).

Purification was achieved by ion exchange chromatography on a DEAE-cellulose column (3 cm × 25 cm) (DE-52, Whatman) equilibrated in 0.02 M tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) in 7 M urea at pH 7.6. The reaction products were dissolved in 5 mL of buffer and 5 mL of 7 M urea, applied to the column, and eluted with the Tris/urea buffer. After elution of unbound material, a

linear gradient (200 mL of buffer and 200 mL of buffer containing 0.05 M NaCl) was employed to elute the shortened relaxin derivative. The protein-containing fractions were desalted on Sephadex G-25 m in 1 M acetic acid and lyophilized. Yields were as follows: fraction 1 = Msc_4-[Hse^{A2}]B29 relaxin, 39.4 mg (19.7%); fraction 2 = Msc_3-des-A(1–2)-B29 relaxin, 74.4 mg (39.9%).

Preparative Edman Degradation. Msc_3-des-Arg,Met-B29 relaxin (155 mg, 25.8 μmol) was suspended in 3 mL of pyridine/water, 9:1. The mixture was flushed with nitrogen for 5 min, and phenyl isothiocyanate (255 μL, 1.3 mmol) was added. The relaxin derivative dissolved during the 3-h reaction at room temperature under nitrogen in the dark. Thereafter, methanol (0.5 mL) was added, and the protein was precipitated with anhydrous ether (15 mL) and centrifuged at 3000 rpm. The pellet was twice resuspended in ether and centrifuged again, the supernatant discarded, and the remaining ether removed in a stream of nitrogen. To remove the excess of reagent, the pellet was kept under high vacuum for at least 1 h. The resulting powder was dissolved in 2 mL of trifluoroacetic acid and was kept under nitrogen in the dark for 1 h. The reaction mixture was then chilled to 0 °C; the protein was precipitated with anhydrous ether and centrifuged. The pellet was twice suspended in ether, centrifuged, and then dried in vacuo. The relaxin was redissolved in 3 mL of 6 M guanidine hydrochloride and separated on a Sephadex G-50 superfine column (3 cm × 54 cm) in 1 M acetic acid containing 0.15 M NaCl. The main peak was desalted on Sephadex G-25 medium in 1 M acetic acid and lyophilized. Msc_3-des-Arg^{A1},Met^{A2},Thr^{A3}-B29 relaxin (98.4 mg) was obtained representing a yield of 66.3%.

Deprotection of Shortened Porcine Relaxin Derivatives and Their Purification. The Msc-protected relaxin derivatives (20 mg) were deprotected in 2 mL of an ice-cold mixture of dioxane/water/2 M NaOH (7:7:2 v/v) for 2 min at 0 °C. The reaction was stopped by addition of 100 μL of acetic acid, and the mixture was separated on a Sephadex G-50 superfine column in 1 M acetic acid (3 cm × 54 cm). Fractions of 5 mL were collected, and fractions 32–37 were pooled and lyophilized. In order to achieve complete deprotection, the procedure had to be repeated once more. Des-A(1–3)-B29, des-A(1–4)-B29, and [Hse^{A2}]B29 relaxins were purified via ion exchange chromatography on a 1.5 × 15 cm CM-cellulose column (CM-52 Whatman) (buffer: 7 M urea, 0.05 M NH_4OAc/AcOH, pH 5.1) with a linear NaCl gradient of 0–0.25 M NaCl (300 mL each), a flow rate of 20 mL/h, and a fraction size of 4 mL. The main peak was desalted on Sephadex G-25 medium in 1 M acetic acid and lyophilized. Des-A(1–5)-B29 and des-A(1–6)-B29 relaxins (1 mg at a time) were purified via reversed-phase high-performance liquid chromatography on a C_{18} (Synchropak RP-P) column (250 mm × 4.1 mm) with a linear gradient of 30% B to 50% B. Solvent A was 0.1% trifluoroacetic acid in water and solvent B 0.1% trifluoroacetic acid in 80% acetonitrile. The main peak was collected, the solvent evaporated in vacuo, and the residue dissolved in 6 M guanidine hydrochloride and desalted on Sephadex G-25 medium in 1 M acetic acid.

Biological Activities

Mouse interpubic ligament assays were peformed according to Steinetz et al. (1960). Immature female ICR mice (20–25 g) were primed with 5 μg of estradiol cyclopentylpropionate in 0.1 mL of sesame oil. Seven days later, the mice (15 per group) were injected with porcine relaxin or porcine relaxin derivative dissolved in 100 μL of a 0.1% solution of benzopurpurin in water. B29 relaxin was used at doses of 0.3, 0.4, 0.5, and 0.8 μg. The same doses of des-A(1–2)-B29, des-A(1–3)-B29, and [Hse^{A2}]B29 relaxins were injected while des-A(1–4)-, des-A(1–5)-, and des-A(1–6)-B29 relaxins were used at doses of 0.3, 0.6, and 3 μg per animal. The mice were killed 12–16 h later in an atmosphere of CO_2. The pubic joint was freed of adhering connective tissue, and the distance between the interpubic bones was measured at 10× magnification on a binocular dissecting scope fitted with an ocular micrometer and transilluminating fiber optics.

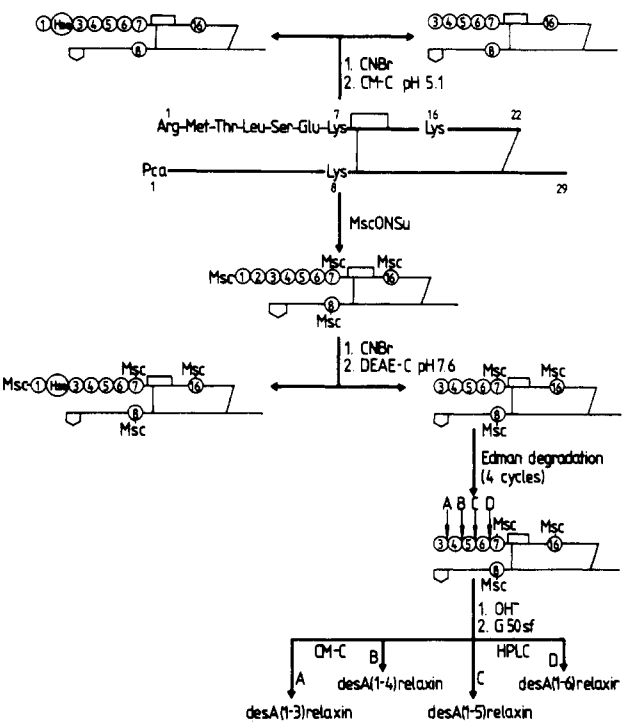

FIGURE 2: Preparation of shortened porcine relaxin derivatives and [Hse^{A2}]B29 relaxin. See text.

Radioimmunoassays of porcine relaxin and porcine relaxin derivatives were performed with a sheep anti-porcine relaxin antibody (S540) in combination with a cellulose-bound rabbit γ-globulin as immunosorbent. [$Formyltyrosyl^{A1}$]relaxin (Schwabe, 1983) iodinated by the chloramine T method (Hunter & Greenwood, 1962) was used as tracer.

Results and Discussion

For a selective reaction at the N terminus of the A chain of porcine relaxin, all ε-amino groups needed to be protected; the N terminus of the B chain of porcine relaxin is blocked by a pyroglutamic acid residue. A single methionine residue in position A2 allowed the initial protection of all four amino groups by the acid-stable Msc group. The required α-amino group is then liberated in position A3 by cyanogen bromide digestion (Figure 2). Further shortening of the porcine relaxin could be accomplished by preparative stepwise Edman degradation.

The amino protecting group could be introduced quantitatively (Büllesbach & Schwabe, 1985c) while the subsequent cyanogen bromide digest of this derivative caused two spots in electrophoresis at pH 8.6, one migrating in the same position as the unmodified Msc-protected relaxin and the other appearing less positive than the starting material. The reaction of Msc_4-B29 relaxin to Msc_3-des-Arg^{A1},Met^{A2}-B29 relaxin leads to the loss of one arginine residue but introduces a new α-amino group. Therefore, the small charge difference is observed only under weakly alkaline conditions.

The separation of both Msc-protected derivatives was possible via ion exchange chromatography on DEAE-cellulose

Table I: Amino Acid Analyses of Shortened Relaxin Derivatives

	B29 relaxin	[Hse^{A2}][a]	des-A(1–2)	des-A(1–3)	des-A(1–4)	des-A(1–5)	des-A(1–6)
Asp	3.19 (3)	2.93 (3)	2.85 (3)	3.35 (3)	3.24 (3)	3.41 (3)	3.36 (3)
Thr	2.12 (2)	2.10 (2)	1.91 (2)	1.08 (1)	1.08 (1)	1.03 (1)	0.88 (1)
Ser	3.36 (4)	4.02 (4)	3.40 (4)	3.50 (4)	3.84 (4)	2.74 (3)	2.60 (3)
Glu	5.66 (5)	5.38 (5)	4.93 (5)	5.49 (5)	5.12 (5)	5.80 (5)	4.30 (4)
Gly	4.15 (4)	4.00 (4)	3.82 (4)	3.59 (4)	3.91 (4)	4.05 (4)	3.95 (4)
Ala	1.64 (2)	1.80 (2)	1.92 (2)	2.14 (2)	2.10 (2)	2.21 (2)	2.11 (2)
Cys[b]	6.49 (6)	5.81 (6)	5.70 (6)	6.44 (6)	6.39 (6)	5.78 (6)	6.14 (6)
Val	4.00 (4)	3.52 (4)	3.51 (4)	3.60 (4)	3.57 (4)	3.76 (4)	3.68 (4)
Met	0.91 (1)	0	0	0	0	0	0
Ile	3.86 (4)	3.62 (4)	3.73 (4)	3.77 (4)	3.41 (4)	4.04 (4)	4.20 (4)
Leu	4.23 (4)	3.78 (4)	3.74 (4)	4.07 (4)	2.98 (3)	2.94 (3)	3.22 (3)
Phe	0.95 (1)	0.81 (1)	0.95 (1)	0.92 (1)	1.00 (1)	0.96 (1)	0.88 (1)
Lys	3.15 (3)	3.35 (3)	3.00 (3)	2.95 (3)	3.08 (3)	3.12 (3)	3.19 (3)
Arg	6.16 (6)	6.00 (6)	5.15 (5)	4.91 (5)	4.68 (5)	5.09 (5)	4.82 (5)
Trp[c]	1.70 (2)						

[a] Homoserine lactone eluted after His but was not quantified. [b] Calculated. [c] Measured by UV spectroscopy.

Table II: Physical and Chemical Properties of Porcine Relaxin with Shortened A Chains or a Modified Methionine Residue

	HPLC		M_r			
relaxin derivative	Msc-protected, t_R (min)	deprotected, t_R (min)	calcd	FAB-MS	calcd A_{282} (cm^2 mg^{-1})	N-terminal sequence
B29 relaxin	52.5	38.5	5813.9	5814.8	1.92	Arg-Met-Thr
des-A(1–2)	51.5	38.0	5526.5		2.02	Thr-Leu
des-A(1–3)	48.5	37.6	5425.4		2.06	Leu
des-A(1–4)	46.8	35.5	5314.0	5358.0	2.10	Ser-Glu
des-A(1–5)	47.1	35.2	5225.1	5243.0	2.14	Glu-Lys
des-A(1–6)	46.0	33.6	5096.0	5114.9	2.19	Lys
[Hse^{A2}]		37.3	5783.9		1.92	Arg-Hse-Thr-Leu

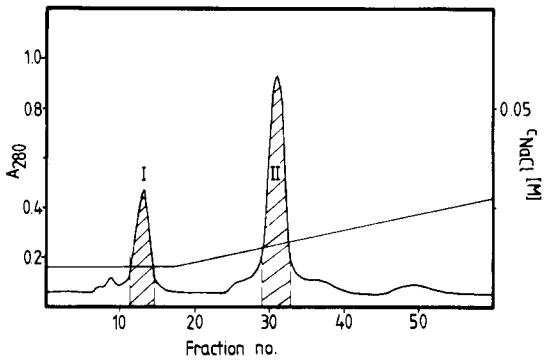

FIGURE 3: Ion exchange chromatography of Msc$_3$-des-Arg,Met-B29 relaxin (II) on a DEAE-cellulose column at pH 7.6 [3 cm × 25 cm; (buffer A) 0.02 M Tris/7 M urea/HCl; (buffer B) buffer A + 0.05 M NaCl; 200 mL each, flow rate 40 mL/h, fraction size 6 mL/tube]. I = Msc$_4$-[Hse^{A2}]B29 relaxin.

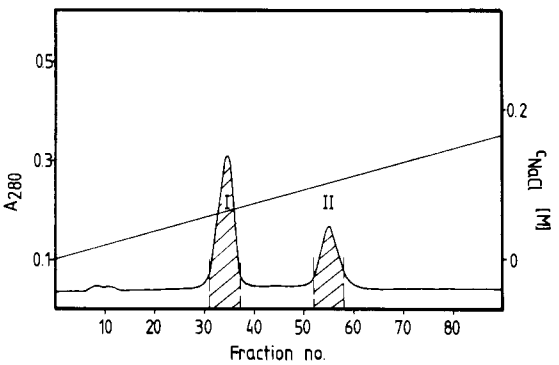

FIGURE 4: CM-cellulose column chromatography of des-Arg,Met-B29 relaxin (I) at pH 5.1 (column 1.5 cm × 20 cm; (buffer A) 0.05 M NH_4OAc/7 M urea/acetic acid; (buffer B) buffer A + 0.2 M NaCl; 200 mL each; flow rate 15 mL/h; fraction size 4 mL/tube]. II = [Hse^{A2}]B29 relaxin.

at pH 7.6 (Figure 3). Amino acid analyses of the crude CNBr digest as well as of the isolated fractions (Table I) showed neither methionine nor methionine sulfoxide. This is consistent with the observation of Schroeder et al. (1969), who suggested that a side reaction occurs during the CNBr digest of Met-Thr sequences whereby the methionine residue is modified to homoserine but the peptide bond is not hydrolyzed. This intramolecular side reaction can be suppressed by using 70% trifluoroacetic acid instead of 70% formic acid (Schroeder et al., 1969).

After CNBr digestion in 70% formic acid, the native porcine relaxin des-Arg,Met-B29 relaxin and [Hse^{A2}]relaxin were obtained in a 1:1 ratio as judged by electrophoresis at pH 4.8, while in 70% trifluoroacetic acid a ratio of about 2:1 was observed. The separation of both compounds could be achieved via ion exchange chromatography on CM-cellulose at pH 5.1 with a linear NaCl gradient (Figure 4). [Hse^{A2}]B29 relaxin was identified by amino acid analysis (Table I) and by sequence analysis of the first four N-terminal residues (Table II). During amino acid analysis, an unknown peak was eluted behind histidine, which corresponded to homoserine lactone. Homoserine was also observed in place of methionine during sequence analysis in the form of its PTH derivative.

Starting with Msc$_3$-des-Arg,Met-B29 relaxin, four residues were removed by manual Edman degradation. After each Edman cycle some higher molecular weight material appeared that could be removed by gel filtration on Sephadex G-50 superfine. The reaction product traveled as a single spot during electrophoresis at pH 4.8 and pH 8.6. The next Edman digest was performed without further purification. The removal of Glu^{A6} after four cycles caused the loss of a negative charge so that the observed electrophoretical purity of the N^{A7},N^{A16},N^{B8}-Msc$_3$-des-A(1–6)-B29 relaxin was a good criterion for a quantitative reaction during the preceding three Edman degradation cycles.

The electrophoretically pure relaxin derivatives showed some impurities in HPLC on reversed-phase columns. The retention times of the main peaks are outlined in Table II. In general, the ϵ-amino-protected shortened relaxins were eluted with a retention time of about 46–53 min.

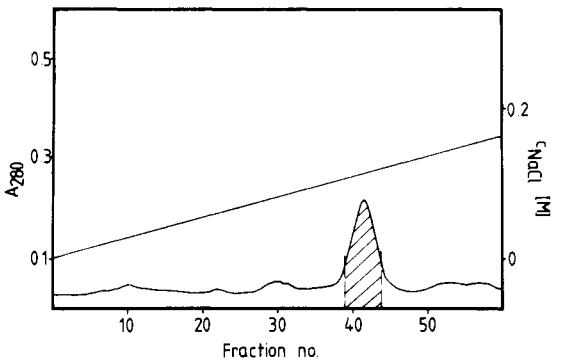

FIGURE 5: CM-cellulose column chromatography of des-A(1–3)-B29 relaxin at pH 5.1 [column 1.5 cm × 20 cm; (buffer A) 0.05 M NH_4OAc/7 M urea/acetic acid; (buffer B) buffer A + 0.2 M NaCl; 200 mL each; flow rate 15 mL/h; fraction size 4 mL/tube].

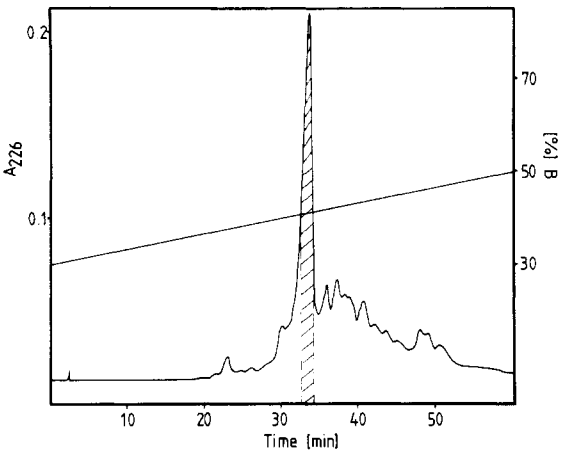

FIGURE 6: HPLC of 1 mg of crude des-A(1–5)-B29 relaxin on a C_{18} reversed-phase column [Synchropak RP-P, 4.1 mm × 250 mm; (solvent A) 0.1% CF_3COOH in water; (solvent B) 0.1% CF_3COOH in 80% acetonitrile; gradient 30–50% B; flow rate 1.5 mL/min].

To ensure that the acid treatment had not caused oxidative damage of the tryptophan side chains, native porcine relaxin was treated with trifluoroacetic acid over 5 h. Neither the UV spectrum nor the mobility on C_{18} columns suggested a destruction of the tryptophan residue.

The shortened relaxin derivatives were obtained after deprotection and purification. The deprotection of the Msc groups was performed in an aqueous alkaline medium for 2 min at 0 °C. A second deprotection step under the same conditions was necessary to remove all protecting groups. This two-step deprotection resulted in a higher purity and a better yield than did one deprotection process extended to 3 or 4 min. After gel filtration, the corresponding porcine relaxin derivative could be purified either by ion exchange chromatography on CM-cellulose at pH 5.1 (Figure 5) or by HPLC (Figure 6). The overall yield was about 10% and was independent of the purification procedure.

All shortened and deprotected relaxins were more than 95% pure as judged by electrophoresis at pH 4.8 and pH 8.6 and HPLC, with a reversed-phase column. The deprotected relaxin eluted 11–14 min faster on a reversed-phase column than did the protected relaxins under identical conditions (Table II). After each cycle, the loss of the corresponding amino acid was demonstrated by amino acid analysis (Table I) and sequence analysis (Table II). The molecular weight of each derivative was determined via fast atom bombardment mass spectrometry, and the experimental values were in good agreement with the theoretically calculated values (Alai et al., submitted for publication).

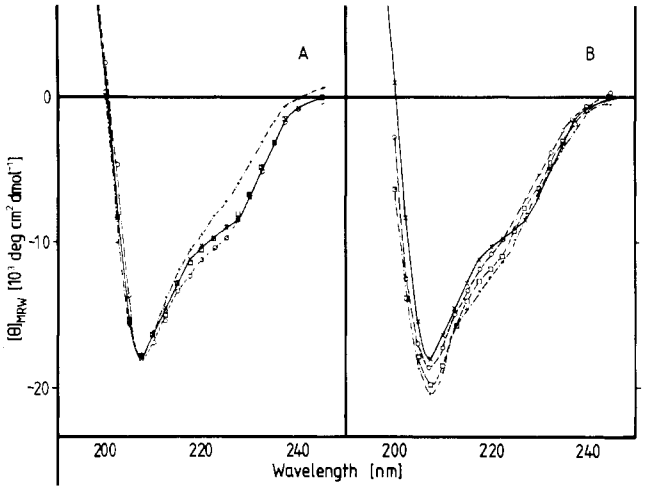

FIGURE 7: CD spectra of (A) (×) B29 relaxin, (□) [Hse^{A2}]B29 relaxin, (○) des-Arg,Met-B29 relaxin, and (+) des-A(1–3)-B29 relaxin and of (B) (×) B29 relaxin, (○) des-A(1–4)-B29 relaxin, (□) des-A(1–5)-B29 relaxin, and (+) des-A(1–6)-B29 relaxin

Investigations of structural changes of the relaxin derivatives were performed by circular dichroism (CD) spectroscopy (Figure 7). As compared to B29 relaxin, none of the derivatives showed a shift of the minimum at 208 nm. The spectrum of [Hse^{A2}]B29 relaxin was identical with that of B29 relaxin, and that of des-Arg,Met-B29 relaxin showed only minor differences (Figure 7A). A change in structure, however, was suggested in des-A(1–3)-relaxin, which showed a significantly less pronounced shoulder at 222 nm. We assume that the shortened N-terminal region no longer permits strong interaction with other portions of the molecule and thus allows water to disrupt the structure in this region. An opposite effect, a more pronounced shoulder at 222 nm, was observed in the case of $N^{\alpha A1}$-citraconylrelaxin (Büllesbach & Schwabe, 1985c). Here, the introduction of a negative charge at the N terminus of the A chain might have caused a salt bridge to form between the protecting group and the side chain of $arginine^{A20}$, resulting in a more restricted structure. With the loss of four, five, or six amino acids from the N terminus of the A chain, the CD spectrum shifted to higher negative values. The more pronounced negative peak at 208 nm, the simultaneous decrease of θ at 200 nm, and the change of the shape of the spectra at about 220 nm indicate a distinct change of the structures as a function of amino acid removal from the A chain.

In radioimmunoassay, [Hse^{A2}]B29 relaxin and des-Arg,-Met-B29 relaxin were equivalent and showed a 50% value of B/B_0 with 2.1 ng/mL, which is not significantly different from the B29 relaxin value. The affinity of shorter relaxins for the antibody decreased as an inverse function of chain length. The slopes of the RIA curves obtained with the des-A(1–5) and des-A(1–6) derivatives differ slightly from the slope obtained from B29 relaxin while all the other relaxin derivatives showed curves parallel to those of B29 relaxin. The affinity appears to be linear with decreasing length of the relaxin A chain except when residue A6 is removed (Figure 8).

The reduction of the antigenecity of des-A(1–6)-relaxin is about 7-fold compared with B29 relaxin and about 3-fold compared with des-A(1–5)-relaxin. We conclude either that an antigenic determinant is located at the N terminus of the A chain or that the loss of the N-terminal segment caused a structural change in the molecule and thereby a reduction of the binding constant for the antigen–antibody complex. To distinguish between both effects, we tested the synthetic heptapeptide Arg-Met-Thr-Leu-Ser-Glu-Lys in the RIA, but no cross-reactivity was observed with concentrations as high

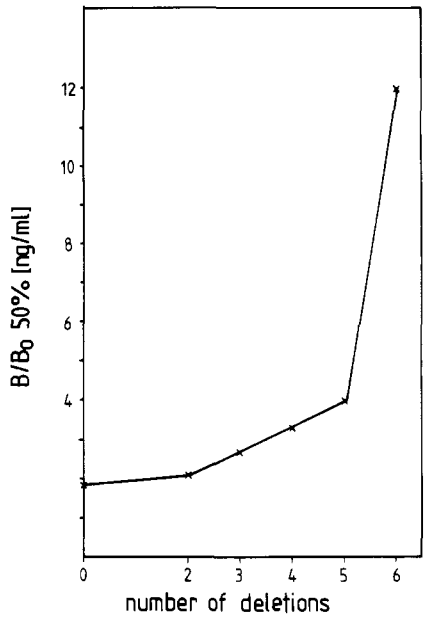

FIGURE 8: Radioimmunoassay of shortened porcine relaxin derivatives: 50% B/B_0 as function of the deletion of amino acid residues from the N-terminal end of the A-chain.

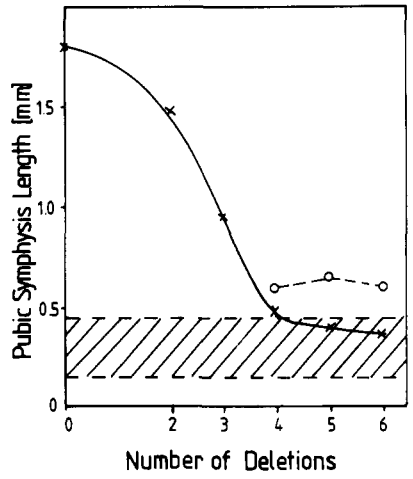

FIGURE 9: Mouse interpubic ligament assay of shortened relaxin derivatives: (×) 0.6 μg of derivative per mouse (mean of 15 mice); (○) 3 μg of derivative per mouse (mean of 15 mice); (hatching) mice injected with benzopurpurine 4B (mean 0.32 mm, standard deviation ±0.18 mm).

as 1 μg/mL. The reduced antibody binding is therefore most likely an effect of structural changes, and the polyclonal antibody used in this assay seems to react with structural features of the relaxin molecule rather than with specific residues.

The biological activity of the derivatives was measured in the mouse interpubic ligament assay (Figure 9) (Steinetz et al., 1960). [Hse^{A2}]relaxin was fully active while des-Arg,-Met-relaxin showed an activity of about 80%. Neither the change of methionine to homoserine in position A2 nor the loss of the first two amino acid residues showed a significant change in the biological potency. However, a drop to 50% bioactivity was found with des-A(1–3)-relaxin, while des-A-(1–4)-, des-A(1–5)-, and des-A(1–6)-relaxins (0.6 μg per mouse) showed no response; concentrations as high as 3 μg per mouse caused minimal widening of the symphysis pubis. The values were far below the half-maximum response of B29 relaxin (0.25 μg/mouse) but slightly higher than the control group of mice without relaxin. The bioactivity of these derivatives is certainty below 5% of the B29 relaxin.

In 1983, Tregear et al. reported the preparation of des-A-(1–3)-relaxin derivatives via total synthesis of the corresponding A and B chains followed by random chain combination. Although the purification data and specific activities of these derivatives have not been published, the authors report some inhibition of the rat uterine contraction to be caused by the chain combination mixture. By comparing the biological activity in the mouse interpubic ligament assay and the structural changes observed in RIA and CD spectroscopy, we were able to demonstrate a simultaneous loss of structure and activity.

Although the des-A(1–3)-relaxin still appears to contain the receptor binding region, it seems to be structurally disturbed so that a full response is no longer demonstrable. In des-A-(1-4)-relaxin and further shortened relaxins it is not clear if the receptor binding region is incompletely present or if the structural changes in the molecule, caused by the removal of amino acids, prohibit receptor binding. Blundell et al. (1983) have postulated that serine in position A5 might be part of the receptor binding region. However, this position varies in human relaxin [human I = Phe (Hudson et al., 1983); human II = Ala (Hudson et al., 1984)] and skate relaxin (Ala) (unpublished results) and is therefore most likely not important.

To elucidate the role of the amino acid residues in the positions A4, A5, and A6, it would be necessary to introduce synthetic peptides in which certain positions of relaxin are exchanged. Such derivatives should recover a more relaxin-like structure, and if so, a more precise statement should be possible about the role of any of the residues that constitute the N-terminal region of the A chain of porcine relaxin.

ACKNOWLEDGMENTS

We are grateful to Dr. Catherine Fenselau, Dr. Robert Cutter, and Mershid Alai, Middle Atlantic Mass Spectrometry Laboratory, Baltimore, MD, for the measurement of the molecular weights by fast atom bombardment mass spectrometry.

REFERENCES

Adler, A. J., Greenfield, N. J., & Fasman, G. D. (1973) *Methods Enzymol. 27*, 675–822.

Blundell, T., Gowan, L. K., & Schwabe, C. (1983) *Int. Congr. Ser.—Excerpta Med. No. 610*, 14–21.

Büllesbach, E. E., & Schwabe, C. (1985a) *Biochem. Biophys. Res. Commun. 126*, 130–135.

Büllesbach, E. E., & Schwabe, C. (1985b) *Biochemistry 24*, 7717–7722.

Büllesbach, E. E., & Schwabe, C. (1985c) *Biochemistry 24*, 7722–7728.

Gowan, L. K., Reinig, J. W., Schwabe, C., Bedarkar, S., & Blundell, T. L. (1981) *FEBS Lett. 129*, 80–82.

Haley, J., Hudson, P., Scanlon, D., John, M., Cronk, M., Shine, J., Tregear, G., & Niall, H. (1982) *DNA 1*, 155–162.

Hudson, P., Haley, J., Cronk, M., Shine, J., & Niall, H., (1981) *Nature (London) 291*, 127–131.

Hudson, P., Haley, J., John, M., Cronk, M., Crawford, R., Haralambidis, J., Tregear, G., Shine, J., & Niall, H. (1983) *Nature (London) 301*, 628–631.

Hudson, P., John, M., Crawford, R., Haralambidis, J., Scanlon, D., Gorman, J., Tregear, G., Shine, J., & Niall, H. (1984) *EMBO J. 3*, 2333–2339.

Hunter, W. H., & Greenwood, F. C. (1962) *Nature (London) 194*, 495–496.

James, R., Niall, H., Kwok, S., & Bryant-Greenwood, G. (1977) *Nature (London) 267*, 544–546.

John, M. J., Borjesson, B. W., Walsh, J. R., & Niall, H. D. (1981) *Endocrinology (Baltimore) 108*, 726-729.
Niall, H. D., John, M., James, R., Kwok, S., Mercado, R., Bryant-Greenwood, G., Bradshaw, R. A., Gast, M., & Boime, I. (1980) in *Insulin: Chemistry, Structure and Function of Insulin and Related Hormones* (Brandenburg, D., & Wollmer, A., Eds.) pp 719-725, de Gruyter, Berlin.
Schroeder, W. A., Shelton, J. B., & Shelton, J. R. (1969) *Arch. Biochem. Biophys. 130*, 551-556.
Schwabe, C. (1983) *Endocrinology (Baltimore) 113*, 814-815.
Schwabe, C., & Braddon, S. A. (1976) *Biochem. Biophys. Res. Commun. 68*, 1126-1132.
Schwabe, C., & McDonald, J. K. (1977) *Science (Washington, D.C.) 197*, 914-915.
Schwabe, C., McDonald, J. K., & Steinetz, B. G. (1976) *Biochem. Biophys. Res. Commun. 70*, 397-405.
Schwabe, C., McDonald, J. K., & Steinetz, B. G. (1977) *Biochem. Biophys. Res. Commun. 75*, 503-510.
Schwabe, C., Anastasi, A., Crow, H., McDonald, J. K., & Barrett, A. J. (1984) *Biochem. J. 217*, 813-817.
Sherwood, O. D. (1979) *Endocrinology (Baltimore) 104*, 886-892.
Steinetz, B. G., Beach, V. L., Kroc, R. L., Stasilli, N., Nussbaum, R., Nemith, P., & Dun, R. (1960) *Endocrinology (Philadelphia) 67*, 102-115.
Tesser, G. I., & Balvert-Geers, I. C. (1975) *Int. J. Pept. Protein Res. 7*, 295-305.
Tregear, G. W., Du, Y. C., Kemp, B., Borgesson, B. W., Scanlon, D., & Niall, H. (1981) in *Relaxin* (Bryant-Greenwood, G. D., Niall, H. D., & Greenwood, F. C., Eds.) pp 151-164, Elsevier/North-Holland, New York.
Tregear, G. W., Du, Y. C., Wang, K. Z., Southwell, C., Jones, P., John, M., Gorman, J., Kemp, B., & Niall, H. D. (1983) *Int. Congr. Ser.—Excerpta Med. No. 610*, 42-55.
Walsh, J. R., & Niall, H. D. (1980) *Endocrinology (Baltimore) 107*, 1258-1260.

Peptide Models of Electrostatic Interactions in Proteins: NMR Studies on Two β-Turn Tetrapeptides Containing Asp–His and Asp–Lys Salt Bridges†

Dinkar Sahal and P. Balaram*
Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India
Received January 29, 1986; Revised Manuscript Received May 1, 1986

ABSTRACT: Two model peptides Boc-Asp-Pro-Aib-X-NHMe [X = His (**1**) and X = Lys (**2**)] were synthesized to simulate intramolecular electrostatic interactions between ionizable side chains. Conformational analysis by 270-MHz ^{1}H NMR in $(CD_3)_2SO$ reveals that the backbone secondary structures of these two peptides are stabilized by two strong intramolecular hydrogen bonds, involving the consecutive carboxy-terminal NH groups. ^{1}H NMR chemical shifts were measured in **1**, **2**, and a protected derivative, Boc-Asp(OBzl)-Pro-Aib-His-NHMe (**3**). These shifts were also measured for the model compounds Ac-Lys-NHMe, Boc-Asp-NHMe, and Boc-His-NHMe in their different states of ionization. An analysis of the chemical shifts of the ionization-sensitive reporter resonances suggests the formation of a strong intramolecular salt bridge in the lysyl peptide **2** and a bridge of moderate strength in the histidyl peptide **1**. A comparison of the temperature dependence of chemical shifts in peptides **1**–**3** suggests that intramolecular salt bridge formation results in diminished backbone flexibility. The results establish that proximity effects confer far greater stability to intramolecular ion pair interactions vis-a-vis their intermolecular counterparts. The salt bridge interaction in peptide **1** displays a remarkable sensitivity to the dielectric constant of the solvent medium. The results suggest that these peptides are good simulators of the role of salt bridges in the structural dynamics of proteins.

Electrostatic ion pair interactions in proteins influence many aspects of their behavior like thermostability, allostery, and enzyme catalysis (Perutz, 1978; Warshel, 1981; Thornton, 1982). Such interactions between amino acid side chains can play a major role in stabilizing specific secondary structures and also in mediating protein–protein interactions (Sundaralingam et al., 1985). The biochemical significance of such interactions may also be implicit in that living cells often respond to external stimuli by reversible posttranslational protein modifications like phosphorylation and methylation (Wold, 1981). These modifications alter the nature and distribution of charges on proteins, which in turn can lead to structural and functional responses.

The intrinsic sensitivity of salt bridges to pH and the fact tha fluctuations in intracellular pH provide a physiological mechanism for regulation of metabolic processes (Roos & Boron, 1981; Busa & Nuccitelli, 1984) and can also modulate intercellular communication by gating gap junctions (Spray & Bennett, 1985) emphasize the importance of these ion pair interactions in fundamental biochemical processes. pH-induced conformational changes can also play an important role in energy transduction in biological systems (Warshel, 1981). Perhaps the most remarkable role of a pH gradient is evident across a lipid bilayer in the conditions under which oxidative phosphorylation occurs (Mitchell, 1961).

While side chains of charged amino acids appear to be best suited to respond to a fluctuating pH, the general distribution of charges on the surface of proteins is often of limited con-

†Supported by a grant from the Department of Science and Technology, Government of India.
*Address correspondence to this author.

0006-2960/86/0425-6004$01.50/0