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Preparation and Properties of α- and ϵ-Amino-Protected Porcine Relaxin Derivatives†

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ABSTRACT: The chemical modification of the amino groups of B29 porcine relaxin resulted in pure derivatives of $N^{\alpha A1}$-citraconyl-B29 relaxin, $N^{\epsilon A7},N^{\epsilon A16},N^{\epsilon B8}$-tris[[[(methylsulfonyl)ethyl]oxy]carbonyl]-B29 relaxin (Msc_3-relaxin), and $N^{\alpha A1},N^{\epsilon A7},N^{\epsilon A16},N^{\epsilon B8}$-tetrakis[[[(methylsulfonyl)ethyl]oxy]carbonyl]-B29 relaxin (Msc_4-relaxin). $N^{\alpha A1}$-Citraconyl-B29 relaxin was obtained after selective deprotection of fully acylated B29 relaxin derivatives. The quantitative reaction of $N^{\alpha A1}$-citraconylrelaxin with [[(methylsulfonyl)ethyl]-oxy]carbonyl succinimide ester followed by deprotection of the citraconyl group resulted in $N^{\epsilon A7},N^{\epsilon A16},N^{\epsilon B8}$-$Msc_3$-B29 relaxin, the starting material for selective chemical modifications at the N terminus of the relaxin A chain. In mouse interpubic ligament assay both Msc_3 and Msc_4 derivatives of relaxin showed a bioactivity of 30%, while in the case of $N^{\alpha A1}$-citraconyl-B29 relaxin the bioactivity was reduced to 15%. When compared with unmodified relaxin, only the circular dichroic spectrum of $N^{\alpha A1}$-citraconyl-B29 relaxin revealed significant differences. Therefore, the loss in bioactivity of the $N^{\alpha A1}$-citraconyl-B29 relaxin seems to be related to the structural changes caused by the introduction of a negative charge at the N terminus of the A chain.

Relaxin, a hormone of pregnancy, consists of two peptide chains held together by disulfide links. Porcine relaxin is variable in the region of the prohormone–hormone junctions at the N terminus of the A chain (Büllesbach & Schwabe, 1985a,b) and at the C terminus of the B chain (Niall et al., 1980; Walsh & Niall, 1980; Büllesbach & Schwabe, 1985b). Since these variations cause no differences in biological activity (Sherwood & O'Byrne, 1974; Tregear et al., 1983; Anderson, 1984; Büllesbach & Schwabe, 1985a), it is reasonable to assume that these regions do not participate in receptor binding.

It is not clear which part of the molecule takes part in hormone–receptor interaction, because (i) no crystal structure could be obtained until now, (ii) relaxin structures of only five different species are known (Schwabe et al., 1976, 1977; James et al., 1977; Gowan et al., 1981; Hudson et al., 1981, 1983, 1984; E. E. Büllesbach et al., unpublished results), and (iii) chemical modification is hampered by the variability of the C-terminal portion of porcine relaxin.

On the basis of the similarity of the disulfide links and other structural features between relaxin and insulin (Schwabe & McDonald, 1977), an insulin-like structure has been predicted for relaxin. Model-building studies (Bedarkar et al., 1977) and computer-graphic studies (Isaacs et al., 1978) have shown that porcine relaxin fits, without strain, into the three-dimensional coordinate system of porcine insulin. Circular dichroism studies of relaxin and insulin also showed a high similarity between both hormones (Schwabe & Harmon, 1978; Rawitch et al., 1980; Du et al., 1982). However, the surface of a relaxin molecule is quite different from the surface of an insulin monomer, and therefore, no biological and immunological cross-reactivities are observed (Rawitch et al., 1980).

On the basis of the insulin-like structure and the primary structure of relaxins of different species, the conserved residues on the surface of the molecule seem to be hydrophilic and basic. Candidates for receptor binding (Figure 1) were described in different papers (Gowan et al., 1981; Bedarkar et

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	1	5	10	15	20
A-chain:	Arg-Met-Thr-Leu-Ser-Glu-Lys-Cys-Cys-Gln-Val-Gly-Cys-Ile-Arg-Lys-Asp-Ile-Ala-Arg-Leu-Cys				
a)			Cys	Gly	Arg Lys Cys
b)					Arg Lys
c)			(Cys)		Arg Lys (Ala) (Cys)
d)		Ser	Lys		Arg Lys

	1	5	10	15	20	25
B-chain	Pca-Ser-Thr-Asn-Asp-Phe-Ile-Lys-Ala-Cys-Gly-Arg-Glu-Leu-Val-Arg-Leu-Trp-Val-Glu-Ile-Cys-Gly-Ser-Val-Ser-Trp-Gly-Arg-					
a)			Cys Gly Arg		Arg	
b)				Arg	Arg	
c)			(Cys)	Arg	Arg(Leu)	(Ile-Cys)
d)			Lys	Arg Glu	Arg	

FIGURE 1: Amino acid residues assumed to be responsible for receptor binding: (a) Bedarkar et al., 1982; (b) Gowan et al., 1981; (c) Dodson et al., 1982; (d) Blundell et al., 1983.

al., 1982; Dodson et al., 1982; Blundell et al., 1983); all of them agree as regards the likely importance of the basic amino acid residues Arg/Lys^{A15}, Lys/Arg^{A16}, Arg^{B12}, and Arg^{B16}.

However, the partial modification of the ϵ-amino groups of the lysines with trinitrobenzenesulfonic acid showed no reduced biological activity in vivo (Schwabe & Braddon, 1976; Schwabe et al., 1978). This is in disagreement with the relevance of a pair of basic amino acids in the A chain (Gowan et al., 1981; Bedarkar et al., 1982; Dodson et al., 1982; Blundell et al., 1983) as well as the supposed importance of the hydrophilic side chains in positions Lys^{A7} and Lys^{B8} (Blundell et al., 1983). The interpretation of these studies is complicated by heterogeneity (1.8 lysines were modified out of 3) and insolubility of N$^{\epsilon}$-modified relaxins, which might have affected the bioactivity.

The isolation of B29 porcine relaxin on a preparative scale (Büllesbach & Schwabe, 1985b) enabled us to produce homogeneous relaxin derivatives. The present paper describes the preparation of N^{α}-citraconyl-B29 relaxin, $N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-Msc$_3$-B29 relaxin,[1] and $N^{\alpha A1}$,$N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-Msc$_4$-B29 relaxin and their chemical and biological properties. $N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-Msc$_3$-B29 relaxin is a suitable starting material for selective elongation or shortening at the N terminus of the A chain.

Materials and Methods

Materials and methods described in the previous paper are not mentioned here. In addition, citraconic acid anhydride was freshly distilled. [[(Methylsulfonyl)ethyl]oxy]carbonyl succinimide ester was prepared according to Tesser & Balvert-Geers (1975). Trypsin (bovine, EC 3.4.21.4) was treated with Tos-PheCH$_2$Cl according to Carpenter (1967).

Tryptic Digestion and Thin-Layer Chromatography. Relaxin (100 μg) or relaxin derivatives were dissolved or suspended in 0.05 M NH_4HCO_3. Trypsin solution (1 μL) (2 μg/μL) in 1 mM HCl was added to start the digest. After 1 h at room temperature, 1 μL of the digest was spotted on precoated cellulose TLC glass plates (Merck, Darmstadt, FRG). Solvent systems used were (a) 1-butanol/pyridine/acetic acid/water (15/10/3/12 v/v), (b) 1-butanol/pyridine/formic acid/water (22/12/1/10 v/v), and (c) 1-pentanol/pyridine/2-butanone/formic acid/water (40/28/11/5.5/14.5 v/v). Spots were developed with 5% (w/v) ninhydrin in 1-butanol/2 N acetic acid, 95/5 v/v.

End-group determination was carried out by one Edman cycle in a Beckman 890 TC automatic sequencer using a 0.1 M quadrol program and polybrene to aid retention of relaxin in the sequencer cup. PTH-amino acids were identified by HPLC (Schwabe et al., 1984). (In the case of citraconyl porcine relaxin, the first cycle was done with a 50-min treatment with heptafluorobutyric acid. The N-terminal amino acid was identified after the second cycle.) End-group determinations via the dansyl technique were performed according to Gray (1967). Dansyl-amino acids were identified by electrophoresis at pH 1.9.

Circular dichroism measurements were performed on a Cary Model 60 CD spectrometer (Cary Instruments, Atlanta, GA) using a 0.05-cm path-length cuvette. Scale expansion of either 0.1 or 0.04° (full range) was selected. Relaxin and Msc-relaxin derivatives were dissolved in 1 mM HCl at a concentration of 1 mg/mL and diluted with the same volume of 0.1 M phosphate buffer, pH 7. Citraconylrelaxin was dissolved at a concentration of 0.5 mg/mL in a 1:1 mixture of 1 mM HCl and 0.1 M phosphate buffer, pH 7. The protein solutions were passed through a Millipore filter (0.45 μm). The protein concentration was determined by UV spectroscopy (A_{282}^{1cm} = 1.92 cm^2 mg^{-1}). The results are presented as mean residual ellipticities according to

$$[\theta] = \frac{\Psi(\mathrm{MRW})}{10CL}$$

where Ψ is the observed rotation, MRW is the mean residual weight, C is the concentration (g/mL), and L is the path length of the cell (cm) (Adler et al., 1973).

Preparation of N^{α}-Citraconyl-B29 Relaxin. B29 relaxin (58 mg, 10 μmol) was suspended in 1 mL of buffer, pH 7 (0.2 M NaH_2PO_4/NaOH). A stock solution of 100 μL of citraconic anhydride in 1.9 mL of dioxane was freshly prepared, and 300 μL (167 μmol) was added to the porcine relaxin suspension. The relaxin mixture turned clear, and 1 to 2 min later a protein precipitation formed where the final pH of the reaction mixture dropped to 4.5. This suspension was incu-

[1] Abbreviations: Msc, [[(methylsulfonyl)ethyl]oxy]carbonyl; A1,A7,A16,B8-Msc$_4$-B29 relaxin, $N^{\alpha A1}$,$N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-tetrakis[[[(methylsulfonyl)ethyl]oxy]carbonyl]-B29 relaxin; A7,A16,B8-Msc$_3$-B29 relaxin, $N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-tris[[[(methylsulfonyl)ethyl]oxy]carbonyl]-B29 relaxin; PTH, phenylthiohydantoin; HPLC, high-performance liquid chromatography; Dns, dansyl; CD, circular dichroism; TLC, thin-layer chromatography.

bated for 14 h at room temperature, and the resulting slightly cloudy solution was adjusted to pH 7–8 with ammonia. The reaction mixture was then dialyzed against water and lyophilized. N^{α}-citraconylrelaxin was separated on CM-cellulose (column 1.5 cm × 20 cm) in 10 mM NH_4HCO_3 with a linear NaCl gradient (0–0.15 M NaCl, 200 mL each). After desalting on Sephadex G-25 m in 10 mM NH_4HCO_3 and lyophilization, 27.4 mg of N^{α}-citraconyl-B29 relaxin was obtained (yield 47.4%).

Preparation of $N^{\epsilon A7}$,$N^{\epsilon A16}$,$N^{\epsilon B8}$-Tris[[[(methylsulfonyl)ethyl]oxy]carbonyl]-B29 Relaxin (A7,A16,B8-Msc$_3$-B29 Relaxin). Procedure I. $N^{\alpha A1}$-Citraconyl-B29 relaxin (60 mg, 10 μmol) was dissolved in 10 mL of dimethyl sulfoxide. Triethylamine (135 μL, 970 μmol) and [[[(methylsulfonyl)ethyl]oxy]carbonyl succinimide ester (30 mg, 113 μmol) were added, and the reaction was stirred for 30 min at room temperature. The mixture was acidified with 1 mL of acetic acid and desalted on Sephadex G-25 m in 1 M acetic acid. The protein-containing fractions were pooled and lyophilized, and the citraconyl group was removed by treatment with 10 mL of 70% formic acid for 5 h at room temperature. After lyophilization, the product was purified on CM-cellulose at pH 4.0 (7 M urea, 0.1 M acetic acid) with a linear NaCl gradient (0–0.1 M NaCl, 250 mL each). The protein-containing fraction was desalted on Sephadex G-25 m in 1 M acetic acid and lyophilized (yield 12.6 mg = 21%).

Procedure II. B29 relaxin (20 μmol, 116 mg) was suspended in 4 mL of buffer, pH 7.0 (0.1 M NaH_2PO_4/NaOH). A solution of citraconic anhydride in dioxane (100 μL of citraconic anhydride in 1.9 mL of dioxane) was freshly prepared, and 600 μL (333 μmol) was added. The suspension turned clear for a short while whereafter a precipitate was observed, most of which redissolved during 14-h reaction at room temperature. Undissolved material was brought into solution by addition of guanidine hydrochloride. The reaction mixture was dialyzed against deionized water (12 h, 3 × 1 L of water), at 4 °C, and lyophilized (yield 107 mg of crude citraconylated relaxin). This material was dissolved in 24 mL of dimethyl sulfoxide, 270 μL (1.840 mM) of triethylamine and 60 mg (126 μmol of [[(methylsulfonyl)ethyl]oxy]carbonyl succinimide ester were added, and the reaction was stirred for 60 min at room temperature and then stopped by adding 2 mL of acetic acid. After desalting on Sephadex G-25 m in 1 M acetic acid and lyophilization, the mixture of relaxin derivatives was redissolved in 10 mL of formic acid (70% v/v).

The deprotection was performed for 5 h at room temperature. Thereafter, the acid was diluted with 100 mL of water and lyophilized. The A7,A16,B8-Msc$_3$-B29 relaxin was separated on CM-cellulose at pH 4.3 (7 M urea/0.1 M acetic acid/ammonia) with a linear NaCl gradient (0–0.15 M NaCl, 300 mL each). Fractions were desalted on Sephadex G-25 m in 1 M acetic acid and lyophilized (yields: 35.5 mg of A7,A16,B8-Msc$_3$-B29 relaxin = 30.4%; 4.9 mg of A1,A7,A16,B8-Msc$_4$-B29 relaxin = 4.2%).

Deprotection of A1,A7,A16,B8-Msc$_4$-B29 Relaxin. Relaxin (10 mg of A1,A7,A16,B8-Msc$_4$) was deprotected in 1 mL of an ice-cold mixture of dioxane/water/2N NaOH (7/7/2 v/v) for 2 min at 0 °C. The reaction was stopped by the addition of 100 μL of acetic acid, and the mixture was separated on Sephadex G-50 sf in 1 M acetic acid containing 0.15 M NaCl (column 3 cm × 54 cm). Fractions 32–37 were pooled and desalted on Sephadex G-25 m in 1 M acetic acid and lyophilized.

Radioimmunoassays and Bioassays. Relaxins (B29 and N^{α}-citraconyl) were dissolved at a concentration of 1 mg/mL.

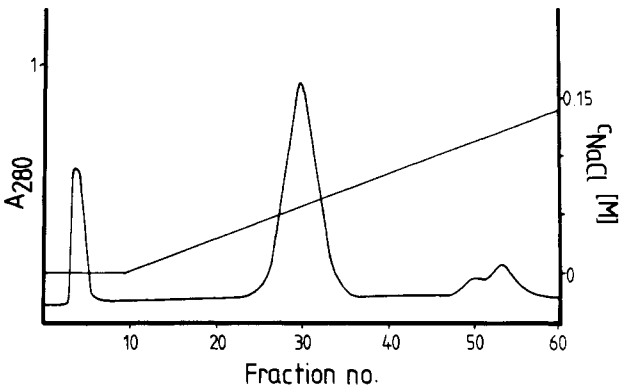

FIGURE 2: Ion-exchange chromatography of crude N-citraconyl-B29 relaxin on CM-cellulose (column, 1.5 × 20 cm; buffer, 10 mM NH_4HCO_3; gradient, 0–0.15 M NaCl, 200 mL each; flow rate, 15 mL/h; fractions, 5 mL/tube). Fractions 5–8, higher substituted citraconyl relaxins; fractions 25–34, N^{α}-citraconyl-B29 relaxin; fractions 52–57, unsubstituted B29 relaxin.

Msc derivatives were dissolved in 1 M acetic acid at a concentration of 2 mg/mL. The acid was neutralized by an equimolar amount of 1 M $NaHCO_3$ solution to a final concentration of 1 mg/mL. The protein content of these stock solutions were checked via UV spectroscopy using the specific extinction coefficient of 1.92 cm^2 mg^{-1}. These stock solutions were used for bioassays and radioimmunoassays. Radioimmunoassays and bioassays were performed as described previously (Büllesbach & Schwabe, 1985b).

Results

Preparation and Properties of N^{α}-Citraconyl-B29 Relaxin. The introduction of the $N^{\alpha A1}$-citraconyl group was achieved by a route similar to that used for the preparation of $N^{\alpha 1}$-citraconylproinsulin (Büllesbach & Naithani, 1980). In an aqueous solution at pH 7 in the presence of 4.2 equiv of citraconic anhydride per amino group two reactions occurred: the acylation of the amino groups and the hydrolysis of free reagent. The latter reaction caused a change of the pH of the reaction solution to pH 4.5 and thus a partial deprotection of the citraconylated relaxin. This reaction can be detected by cellulose–acetate electrophoresis at pH 8.6. The higher substituted relaxins precipitated under these pH conditions. During the incubation overnight the precipitate redissolves under partial deprotection.

The most labile protecting groups are the N^{ϵ}-citraconyl groups of the lysines. Therefore, the liberation of the ε-amino groups is preferred under the chosen pH conditions. The $N^{\alpha A1}$-citraconyl group is more stable but not resistant under these conditions.

$N^{\alpha A1}$-Citraconyl-B29 relaxin was separated by ion-exchange chromatography on CM-cellulose at pH 8.2 with a linear NaCl gradient (Figure 2). Monosubstituted relaxin was eluted in a single peak in a yield of 47.4%. A small amount of an impurity and unmodified porcine relaxin was eluted with a higher salt concentration; the higher substituted relaxins were not bound to the CM-cellulose at all.

At pH 8.6 a difference of one negative charge could be observed between B29 relaxin and N^{α}-monosubstituted relaxin and of two negative charges between B29 relaxin and N^{ϵ}-monosubstituted relaxin. Electrophoretical studies of the reaction mixture showed no band in the position of an N^{ϵ}-monosubstituted citraconylrelaxin while during investigations of the break-through peak from the CM-cellulose column (Figure 2) a small band could be demonstrated in the position of N^{ϵ}-citraconylrelaxin. According to these observations, the

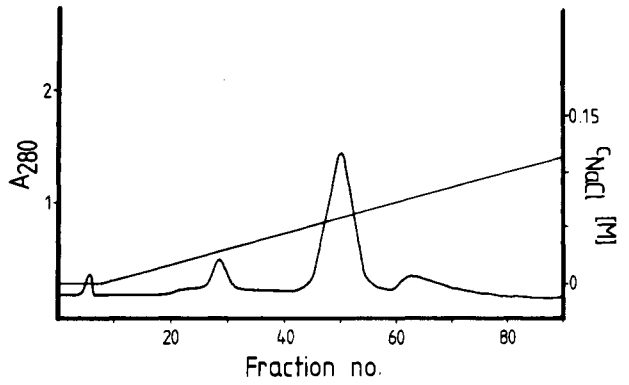

FIGURE 3: Ion-exchange chromatography of crude Msc_x-B29 relaxin derivatives on CM-cellulose (column, 3 × 25 cm; buffer, 7 M urea/0.1 M acetic acid/ammonia, pH 4.3; gradient, 0–0.1 M NaCl, 300 mL each; flow rate, 50 mL/h; fractions, 5 mL/tube). Fractions 27–31, A1,A7,A16,B8-Msc_4-B29 relaxin; fractions 45–54, A7,A16,B8-Msc_3-B29 relaxin.

N^{ϵ}-monosubstituted citraconylrelaxin might be present in amounts of less than 3%.

The isolated $N^{\alpha A1}$-citraconylrelaxin migrated in a single spot in electrophoresis at pH 8.6. End-group determination was performed via two cycles of automatic Edman degradation. $N^{\alpha A1}$-Citraconyl-B29 relaxin has no free α-amino group, and consequently, no amino acid could be found in the first cycle of the Edman degradation. After treatment with heptafluorobutyric acid (50 min) during the first cycle, the acid-labile protecting group was removed, and the second cycle yielded PTH-arginine (but no PTH-methionine) in the expected amount. This result was confirmed by the end-group determination using the dansyl technique (Gray, 1967), which yielded only free N^{ϵ}-Dns-lysine.

Preparation and Properties of A7,A16,B8-Msc_3-B29 Relaxin. To protect all three of the ϵ-amino groups of the citraconylated derivative, two different routes were chosen. The first procedure started with pure $N^{\alpha A1}$-citraconylrelaxin, the quantitative introduction of the Msc-protecting groups followed by liberation of the α-amino group by treatment with 70% formic acid over 5 h at room temperature. A7,A16,B8-Msc_3-B29 relaxin was separated via ion-exchange chromatography on CM-cellulose at pH 4.0 in 7 M urea. The derivative was obtained in a yield of 21%, corresponding to an overall yield of about 10% based on B29 relaxin starting material.

The second procedure was based on the facts (i) that the reaction mixture of citraconylrelaxin consists mainly of N^{α}-monosubstituted, N^{α},N^{ϵ}-disubstituted, and unsubstituted relaxin and (ii) that the introduction of the Msc protecting groups is quantitative. The crude citraconylated mixture of relaxin was reacted with [[(methylsulfonyl)ethyl]oxy]carbonyl succinimide ester. The reaction was stopped with acetic acid and the protein desalted on Sephadex G-25 m in 1 M acetic acid. During this procedure, removal of some of the citraconyl groups took place. However, 1 M acetic acid is not strong enough for a quantitative liberation of the α-amino group. The lyophilized relaxin mixture was therefore treated with 70% formic acid over 5 h at room temperature to completely remove the citraconyl group. The Msc-protected relaxins were separated on CM-cellulose at pH 4.3 (Figure 3). The A7,A16,B8-Msc_3-B29 relaxin was isolated in an overall yield of 30.4%. The corresponding A1,A7,A16,B8-Msc_4-B29 relaxin was obtained in a yield of 4.2%. The A7,A16,B8-Msc_3-B29 relaxin derivatives synthesized by these two different methods were identical in their electrophoretic mobility in cellulose-acetate electrophoresis at pH 4.8 and pH 8.6. Compared with the isolated A1,A7,A16,B8-Msc_4-B29 relaxin, the Msc_3-B29 relaxin migrated faster to the cathode at pH 4.8 but had a lower negative charge at pH 8.6. The latter observation is probably explained by the fact that some protonation of the free α-amino group occurs in A7,A16,B8-Msc_3-B29 relaxin.

A trisubstituted Msc-relaxin with one free ϵ-amino acid group would show a different migration in electrophoresis at pH 8.6. Because such a spot was not found, we assumed a pure A7,A16,B8-Msc_3-B29 relaxin with the free amino group in position A1. This was confirmed by end-group determination via Edman degradation, which resulted in the theoretically expected amount of PTH-arginine. By use of the dansyl technique, Dns-Arg but not N^{ϵ}-Dns-Lys could be identified by electrophoresis (Gray, 1967). A1,A7,A16,B8-Msc_4-B29 relaxin showed only a minor peak of Dns-Arg and no N^{ϵ}-Dns-Lys by this technique. The amount of free N-terminal arginine was determined by Edman degradation to be less than 4%. Tryptic digestion was performed, and the corresponding peptide mixture was separated on thin-layer chromatography in three different systems (Figure 4). Some tryptic peptides of native B29 relaxin were isolated via reversed-phase HPLC (data not shown) and identified by amino acid analysis, and their migration in thin-layer chromatography was investigated. The tryptic peptides of the Msc-protected relaxins contained neither the peptide B(1–8) nor A(2–7), nor Lys^{A16}. In the case of A7,A16,B8-Msc_3-B29 relaxin, the N-terminal arginine was observed whereas in A1,A7,A16,B8-Msc_4-B29 relaxin this amino acid was missing.

The purity of the A7,A16,B8-Msc_3-B29 relaxin and the A1,A7,A16,B8-Msc_4-B29 relaxin was checked by reversed-phase HPLC (Figure 5). Relaxin and relaxin derivatives were eluted in the order B29 ≫ Msc_3-B29 relaxin > Msc_4-B29 relaxin, which is in harmony with the expected changes in hydrophobicity. Both Msc derivatives are not absolutely pure under HPLC conditions. For biological and physicochemical studies, both derivatives were further purified by HPLC with 0.1% trifluoroacetic acid and a gradient of acetonitrile. The solvent was removed in vacuo. To remove traces of acetonitrile and the trifluoroacetate counterions, the relaxin derivatives were desalted on Sephadex G-25 m in 1 M acetic acid.

Deprotection of A1,A7,A16,B8-Msc_4-B29 Relaxin. The deprotection of A1,A7,A16,B8-Msc_4-B29 relaxin was investigated under similar conditions as described for insulin (Geiger et al., 1975) but with a 2-min reaction time. The deprotected relaxin was purified on Sephadex G-50 sf in 1 M acetic acid/0.15 M NaCl. As judged by electrophoresis at pH 4.8, it appears that 85–90% of the relaxin is regenerated, 10% is monosubstituted, and less than 5% disubstituted relaxins were present. These impurities could be separated by reversed-phase HPLC, resulting in a relaxin identical with native B29 relaxin in electrophoresis at pH 4.8 and pH 8.6, in HPLC on reversed phase, and after tryptic digestion and thin-layer chromatography of the peptides in three different systems (Figure 4).

The Msc group as a semipermanent protecting group seems to be more stable in relaxin than is described for insulin. With a reaction time of 2 min, a minor disulfide exchange caused the formation of a small amount of polymers. Further extension of the reaction time to 4 min showed a higher degree in deprotection and a distinct increase in disulfide exchange.

Circular Dichroism. B29 relaxin, $N^{\alpha A1}$-citraconylrelaxin, A7,A16,B8-Msc_3-B29 relaxin, and A1,A7,A16,B8-Msc_4-B29 relaxin and its deprotected product were investigated by circular dichroic spectroscopy (Figure 6). The spectra of B29 relaxin, the deprotected Msc_4-relaxin, A7,A16,B8-Msc_3-B29 relaxin, and A1,A7,A16,B8-Msc_4-B29 relaxin are very similar

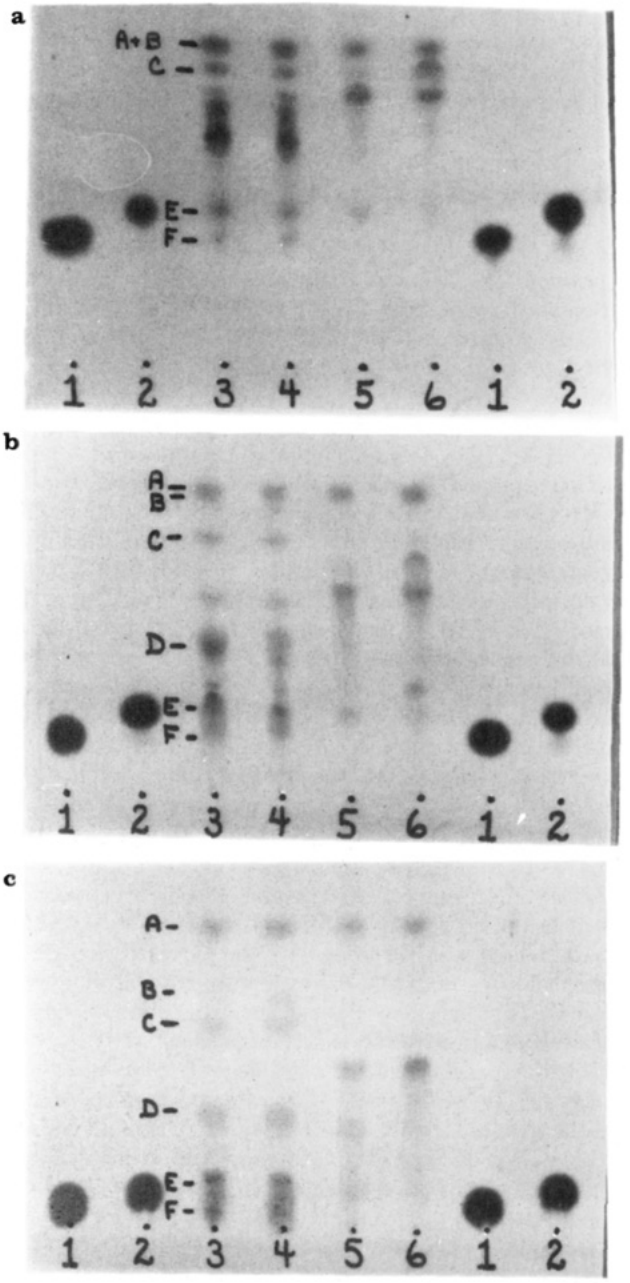

FIGURE 4: Thin-layer chromatography of tryptic digests on precoated cellulose TLC plates in the following solvent systems: (a) 1-butanol/pyridine/acetic acid/water, 15/10/3/12; (b) 1-butanol/pyridine/formic acid/water, 22/12/1/10; (c) 1-pentanol/pyridine/2-butanone/formic acid/water, 40/28/11/5.5/14.5. (1) Lys; (2) Arg; (3) B29 relaxin; (4) deprotected A1,A7,A16,B8-Msc_4-B29 relaxin; (5) A7,A16,B8-Msc_3-B29 relaxin; (6) A1,A7,A16,B8-Msc_4-B29 relaxin. Peptides identified were (A) B(13–16), (B) B(1–8), (C) A(2–7), (D) A(17–20), (E) Arg, and (F) Lys.

Table I: Biological and Physical–Chemical Properties of Relaxin Derivatives

derivative	bioassay (%)	RIA (%)	$\theta_{208}/\theta_{222}$
B29 relaxin	100	100	1.77
A1-citraconyl-B29 relaxin	15	100	1.53
A7,A16,B8-Msc_3-B29 relaxin	30	100	1.80
A1,A7,A16,B8-Msc_4-B29 relaxin	30	100	1.73
Msc_4-B29 relaxin deprotected	100	100	1.80
[Phe^{A0}]B29 relaxin[a]	100	100	1.75

[a] Büllesbach & Schwabe, 1985a.

with a minimum at 208 nm and a shoulder at 222 nm. The ratio $\theta_{208}/\theta_{225}$ outlined in Table I shows only a minor difference between these derivatives with a value of 1.77 for B29 relaxin.

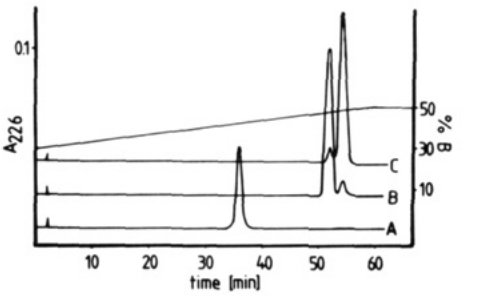

FIGURE 5: HPLC of (A) B29 relaxin, (B) A7,A16,B8-Msc_3-B29 relaxin, and (C) A1,A7,A16,B8-Msc_4-B29 relaxin (column, Synchropak RP-P; solvent system A, 0.1% trifluoroacetic acid in water; solvent system B, 0.1% trifluoroacetic acid in 80% acetonitrile; linear gradient, 30–50% B in 1 h; flow rate, 1.5 mL/min).

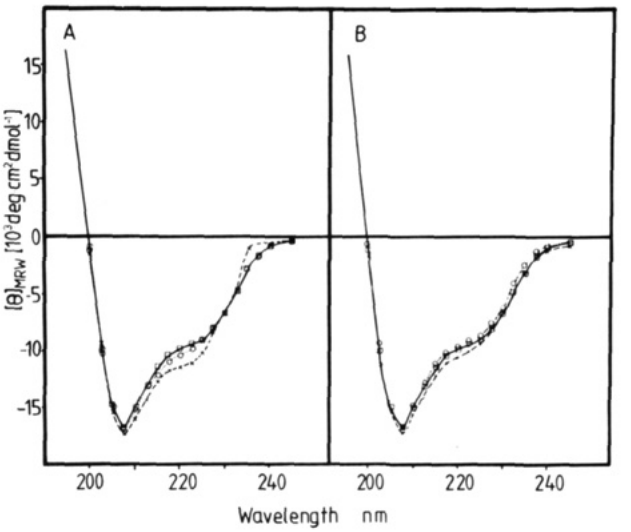

FIGURE 6: CD spectra of (A) B29 relaxin (□), A7,A16,B8-Msc_3-B29 relaxin (○), and N^{α}-citraconyl-B29 relaxin (×) and of (B) B29 relaxin (□), A1,A7,A16,B8-Msc_4-B29 relaxin (×), and A1,A7,A16,B8-Msc_4-B29 relaxin deprotected (○).

Du et al. (1982) described a $\theta_{210}/\theta_{225}$ at 1.69 for the same derivative, and Schwabe & Harmon described a $\theta_{208}/\theta_{222}$ of 1.66 for a native pig relaxin preparation.

In contrast, a distinctly different CD spectrum was found in the case of $N^{\alpha A1}$-citraconyl-B29 relaxin resulting in a low $\theta_{208}/\theta_{222}$ ratio. The minimum at 208 nm is similar to B29 relaxin, whereas the minimum at 222 nm is much more pronounced.

Immunological and Biological Activities. For bioassay and radioimmunoassay, relaxin and $N^{\alpha A1}$-citraconyl-B29 relaxin were dissolved in water. In the case of the Msc-relaxin derivatives, solution was achieved in 1 M acetic acid. When the acid was neutralized with equimolar amounts of $NaHCO_3$, the Msc-relaxin derivatives remained in solution.

With polyclonal antibodies there was no distinct difference between native and amino-protected relaxin derivatives (Table I). In the mouse interpubic ligament assay all amino-protected relaxins showed some decreased bioactivity (Table I). The activity of the Msc-protected B29 relaxin was about 30%, while the activity of the $N^{\alpha A1}$-citraconyl-B29 relaxin dropped to around 15% of native relaxin. The deprotection of A1,A7,A16,B8-Msc_4-B29 relaxin resulted in a fully biologically active relaxin.

Discussion

The preparation of A7,A16,B8-Msc_3-B29 relaxin was made possible by the intermediary preparation of the $N^{\alpha A1}$-citraconyl-B29 relaxin. This relaxin derivative consists of only

one free α-amino group and can be used for either selective shortening or extension at the N terminus of the A chain. The application of the Msc group has the advantages that this group is hydrophilic and is stable under acid conditions, which will allow the use of condensations of active esters and the use of Edman degradation. The Msc derivatives showed a very high solubility in aqueous acidic solutions (<pH 4), and no precipitation occurred after change of pH to 7. This made possible investigations of the bioactivity and the circular dichroism of Msc-protected derivatives and the $N^{\alpha A1}$-citraconyl-B29 relaxin under the same conditions.

Investigations of the structure and function of the partially protected relaxin molecules led to surprising results. The introduction of four and three neutral amino-protecting groups did not cause as dramatic a drop in bioactivity as did the introduction of the one negatively charged citraconyl group in position A1. Since there is no significant difference in the bioactivity of A7,A16,B8-Msc_3-B29 relaxin and A1,A7,A16,B8-Msc_4-B29 relaxin, this change is not likely due to the N^{α} protection itself. For instance, no change in bioactivity is observed by the N-terminal elongation of the A chain by either an amino acid like phenylalanine (Büllesbach & Schwabe, 1985a) or an amino-protected amino acid like formyltyrosine (Schwabe, 1983). The only plausible explanation remaining is that the introduction of the negative charge at the N terminus of the A chain caused the decrease in bioactivity. Comparison of the circular dichroism of B29 relaxin and $N^{\alpha A1}$-citraconyl-B29 relaxin suggests a change in the three-dimensional structure, which in turn might have caused the loss in bioactivity.

On the basis of the model of porcine relaxin (Bedarkar et al., 1977; Isaacs et al., 1978), the N terminus of the A chain and the C terminus of the A chain form α-helical segments. Because the midregion of the A chain forms a U-turn, both helical segments are close together whereas electrostatic repulsion between the N-terminal amino group of the A chain and the guanidine side chain of Arg^{A1} and of Arg^{A20} might keep both helical segments at a certain distance. The modification of the α-amino group by neutral protecting groups or an N-terminal elongation would not disturb this arrangement. Thus, the introduction of the negatively charged citraconyl group at the N terminus causes the formation of a salt bridge between the protecting group and the guanidine side chain of Arg^{A20}, thereby restricting flexibility in the terminal helix region. Such loss in mobility is commensurate with a stronger 220-nm signal in the CD spectrum.

Investigations of the circular dichroism of A7,A16,B8-Msc_3-B29 relaxin and A1,A7,A16,B8-Msc_4-B29 relaxin resulted in spectra similar to those of B29 relaxin. This finding does not exclude the possibility of minor changes in the structure that can cause a reduced bioactivity. The remaining bioactivity of both Msc-relaxin derivatives of about 30% together with the fully biologically active relaxin derivatives partially modified at the amino groups described by Schwabe & Braddon (1976) makes it unlikely that the lysines play a very important role in the hormone–receptor interaction and (by steric arguments) diminishes substantially a possible role of Arg^{A15}. To be able to answer questions concerning the location of the receptor binding site of relaxin, much more information is needed, including intensive physical-chemical and biological investigations of new synthetic derivatives.

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Registry No. $N^{\alpha A1}$-Citraconyl-B29 relaxin, 98921-26-7; A7,A16,B8-Msc_3-B29 relaxin, 98921-27-8; A1,A7,A16,B8-Msc_4-B29 relaxin, 98921-30-3; [Phe^{A0}]B29 relaxin, 95506-55-1; B29 relaxin, 77467-85-7; [[(methylsulfonyl)ethyl]oxy]carbonylsuccinimide ester, 75189-87-6; citraconic anhydride, 616-02-4.

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Mapping of Gonadotropin-Releasing Hormone Receptor Binding Site†

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ABSTRACT: On the basis of the spatial conformation of gonadotropin-releasing hormone (GnRH), we have predicted that aromatic amino acids and at least one carboxyl group are involved in the recognition site of the receptor. Therefore, various specific reagents were examined for their ability to interfere with the binding of GnRH to its receptor. Pretreatment of pituitary membrane preparations with sodium periodate decreased the specific binding in a dose-dependent manner (IC_{50} = 0.5 mM) due to a decrease in receptor affinity. This indicated the presence of a sugar moiety in the binding site. Tryptophan is another constituent that participates in the GnRH binding site, as pretreatment of pituitary membranes with 2-methoxy-5-nitrobenzyl bromide inhibited the binding (IC_{50} = 0.22 mM) by decreasing receptor affinity. In addition, the native hormone conferred on the binding site a protective effect against inactivation by 2-methoxy-5-nitrobenzyl bromide. Pretreatment of membranes with *p*-diazobenzenesulfonic acid also inhibited the binding of ^{125}I-Buserelin (IC_{50} = 0.1 mM), indicating the presence of tyrosine within or near the binding site. Pretreatment of pituitary membrane preparations with dithiothreitol also inhibited the binding due to a decrease in the binding affinity, which was accompanied by an increase in receptor number. These data suggest that there are disulfide bonds within or near the binding region. Treatment with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide and glycine ethyl ester also prevented binding in a dose-dependent manner and implies that free carboxylic groups are involved in the binding site. Since divalent cations were more potent in inhibiting the specific binding in comparison with monovalent cations and since the order of potency within the divalent cations was identical with their association constants to dicarboxylic compounds, it is suggested that there are at least two carboxylic groups that participate in the binding of the hormone. According to these findings, we propose a model describing the interaction involved in the formation of the hormone–receptor complex.

The secretion of gonadotropins from the pituitary gland is stimulated by the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH).[1] The first step in GnRH action is its interaction with specific receptors at the surface of gonadotrope cells (Conn et al., 1981). Photoaffinity labeling of the GnRH receptors of rat pituitary membrane preparations resulted in the identification of a single specific band with an apparent M_r of 60K (Hazum, 1981a,b; Hazum & Keinan, 1983). Treatment of rat pituitary membranes with trypsin, chymotrypsin, and neuraminidase resulted in a decrease of the specific binding of iodinated GnRH agonist and antagonist (Hazum, 1981c, 1982). These results indicated that the GnRH receptor is a glycoprotein. Moreover, preincubation of membrane preparations with wheat germ agglutinin, a specific lectin for sialic acid and *N*-acetylglucosamine, caused a marked inhibition of GnRH agonist and antagonist binding, suggesting that sialic acid is an integral component of the GnRH receptor, essential for binding (Hazum, 1982). Digestion of pituitary membranes with various phospholipases reduced the specific binding of both GnRH agonist and antagonist, implicating membrane phospholipid involvement in the hormone–receptor interaction (Hazum et al., 1982). In this study we have used specific chemical reagents to analyze the components comprising the binding site, essential for the recognition of the hormone.

Materials and Methods

Materials

All reagents used were purchased from the usual commercial sources.

Methods

Iodination and Pituitary Membrane Preparations. [D-Ser(Bu^t)^6,des-Gly^{10},ethylamide]GnRH (Buserelin, GnRH agonist provided by Dr. J. Sandow, Hoechst) was iodinated by the lactoperoxidase method (Sandow & Konig, 1979). Specific activity of the labeled peptide was approximately 1.0 mCi/μg, as measured by self-displacement in the pituitary radioligand receptor assay. Pituitary membranes were prepared from 25 to 28 day old Wistar-derived female rats according to Heber & Odell (1978), with modification. Briefly, the glands were homogenized gently with a tight Dounce homogenizer at 4 °C in assay buffer [10 mM Tris-HCl, pH

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[1] Abbreviations: GnRH, gonadotropin-releasing hormone; Buserelin, [D-Ser(Bu^t)^6,des-Gly^{10},ethylamide]GnRH; DTT, dithiothreitol; GEE, glycine ethyl ester; DCI, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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