Vol. 126, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1985 Pages 130-135

[A0-PHENYLALANYL] RELAXIN (PORCINE): AN ACTIVE INTERMEDIATE

Bullesbach, E.E. and Schwabe, C.

Department of Biochemistry
Medical University of South Carolina
Charleston, SC 29425

Received November 15, 1984

SUMMARY: A [phenylalanyl^{A0}] relaxin has been isolated as a byproduct during large scale porcine relaxin preparations, using ion exchange chromatography on CM-cellulose at pH 7.8 followed by high performance liquid chromatography on reversed phase columns. The elongation at the N terminus of the A-chain has been demonstrated by amino acid and sequence analyses of the isolated and carboxymethylated relaxin-A-chain. The phenylalanyl relaxin and B29 relaxin are indistinguishable by circular dichroism spectroscopy, in mouse pubic ligament assay, and radioimmunoassay. The occurrence of phenylalanyl relaxin may be caused by an incomplete conversion of prorelaxin to relaxin.

The peptide hormone relaxin (M_r = 6000) consists of two polypeptide chains (1,2,3), linked by disulfide bonds (4) (Fig. 1). Isolation of relaxin from pregnant sow ovaries via ion exchange chromatography has resulted in three compounds with identical bioactivity (5). These relaxin molecules differ in length at the C terminus of the B-chain from 28 to 31 amino acid residues, whereas the length of the A-chain is constant at 22 amino acid residues (6). An essentially homogeneous porcine relaxin was isolated by a modified extraction procedure (7), and relaxin with 31 amino acids in the B-chain was identified as the predominant form of the hormone in pig ovaries. Shorter forms (B-chain) could have been produced during the isolation procedure (7).

However, it is still unclear where the initial enzymatic processing sites are located in the B-chain/C-peptide junction as well as in the C-peptide/A-chain junction. Haley et al. (8) consider different possibilities, including the direct conversion to B31 and/or B32 relaxin by a chymotrypsin-like enzyme, or the formation of [Leu-Phe^{A0}]B31 (or B32)

Abbreviations: EDTA = ethylenediamine tetraacetate; HPLC = high performance liquid chromatography; PTH = phenylthiohydantoin; tris = tris(hydroxymethyl)aminomethane

0006-291X/85 $1.50

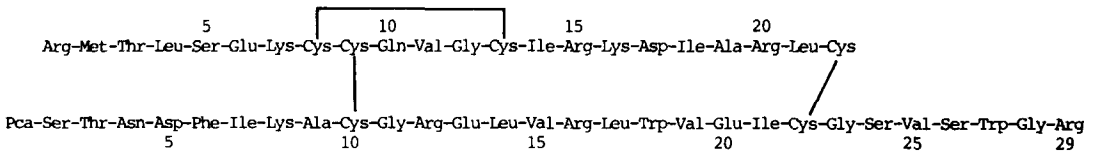

Fig. 1: Primary sequence of B29 porcine relaxin

relaxin by a combination of a chymotrypsin- and trypsin-like enzyme. In the last case the Leu-Phe extension at the N terminus of the A-chain and Leu^{B32} at the C terminus of the B-chain must be removed by exopeptidases. The processing to [Leu-Phe^{A0}]B32 relaxin would require the same combination of enzymes as the conversion of rat prorelaxin to rat relaxin (8). From this point of view natural products of porcine prorelaxin conversion could be obtained as intermediate forms which do not only vary at the C terminus of the B-chain, but also at the N terminus of the A-chain. In this paper we describe the isolation and characterization of [Phe^{A0}] porcine relaxin as a fully active intermediate of porcine prorelaxin conversion.

MATERIALS AND METHODS: Relaxin was purified from an acetone-HCl extract by ion exchange chromatography on CM-cellulose at pH 5.5, using a stepwise gradient of 50 mM ammonium acetate buffer in 0, 0.2, and 0.5 M NaCl. The relaxin, contained in the 0.2 M NaCl fraction, was further purified via ion exchange chromatography on CM-cellulose at pH 7.8 (7 M urea/50 mM tris/HCl) using a linear NaCl-gradient (0-0.1 M NaCl). B29 relaxin and further elongated relaxins were eluted in a double peak. After carboxypeptidase A digestion of the pooled double peak (1 h, 37°C, in 50 mM NH_4HCO_3, enzyme substrate ratio 1:50) and separation on Sephadex G 50 sf in 1 M acetic acid containing 0.15 M NaCl, the resulting B29 relaxin was rechromatographed on CM-cellulose at pH 7.8. B29 relaxin was eluted in the main peak while a side product eluted earlier. This side product was further purified via HPLC on reversed phase.

High-performance liquid chromatography was performed on a Waters chromatograph (Waters Associates, Milford, MA) equipped with a Uvicord S photometer (LKB, Bromma, Sweden) (226 nm), a model 660 solvent gradient programmer (Waters), and a Synchropak RP-P column (250 mm x 4.1 mm, Synchron Inc., Linden, IN). The solvent systems used were: A) 0.1% trifluoroacetic acid in water, and B) 0.1% trifluoroacetic acid in 80% acetonitrile. A linear gradient of 60 min was employed from 30% B to 50% B at a constant flow rate of 1.5 ml/min. Peptide mixtures of 0.5 mg - 1 mg were separated and the pooled fractions of repeated runs rechromatographed under the same conditions. The purity was confirmed by using 0.02 mg of the material in an analytical run on the same octadecyl silane column.

Amino acid analyses were performed on a Durrum D 502 (Durrum Instrument Co., Palo Alto, CA) analyzer after 24 h of hydrolysis in vacuo in 6 N HCl at 120°C. Sequence analysis was carried out by Edman degradation in a Beckman 809 TC automatic sequencer using a 0.1 M quadrol program and polybrene to aid retention of the relaxin A-chain in the sequencer cup. PTH amino acids were identified by HPLC (9). Ultraviolet spectra were measured with a Cary 15 recording spectrophotometer (Cary Instruments, Atlanta, GA). Protein concentrations of relaxin were calculated using a specific absorption coefficient of a 0.1% solution: A(282) = 1.92.

Circular dichroism measurements were made on a Cary model 60 CD spectrophotometer (Cary Instruments, Atlanta, GA), using a cell of 0.05 cm pathlength. A

scale expansion of 0.04 degree (full range) was selected. Relaxin and relaxin derivatives were dissolved in 0.001 M HCl and diluted with the same amount of 0.1 M phosphate buffer (pH 7.0) to a concentration of 30 µM to 60 µM. The results are represented as mean residue ellipticities [Θ] according to the expression:

$$[\Theta]_n = \frac{[\psi]\ [MRW]}{10\ cl}$$

where ψ is the observed rotation (degree), MRW is the mean residual weight (g/mol), c is the concentration in g/ml, and l is the pathlength of the cell in cm (10).

Cellulose acetate electrophoresis was carried out in a Deluxe electrophoresis chamber (Gelman Science, Ann Arbor, MI). The cellulose acetate strips used (Sephaphor III (2.5 cm x 17.1 cm)) were a product of the same company. Two different electrophoresis systems were employed, one at pH 4.8 (4 M urea, 0.1 M pyridine adjusted to pH 4.8 with acetic acid for 2 h at 200 V), and the other one at pH 8.6 (4 M urea, 25 mM sodium barbital, 25 mM sodium acetate, 5 g/l EDTA, 10 vol % dioxane) for 3 h at 200 V. Strips were stained in 0.2% Ponceau S in 3% trichloroacetic acid.

For the radioimmunoassay of relaxin sheep anti-porcine relaxin antibody in combination with a goat anti sheep- -globulin immunoabsorbent cellulose was used. Iodinated formyltyrosyl relaxin (11) was used as tracer.

Mouse pubic ligament assays were performed according to Steinetz et al. (12) in immature female IRC mice, which had been primed 5 days prior with 5 µg estradiol cyclopentylpropionate in 0.1 ml sesame oil. The doses of relaxin were 0.3 µg and 0.6 µg per mouse, respectively, injected in 0.1 ml 1% Benzopurpurin 4B.

RESULTS AND DISCUSSIONS: Carboxypeptidase A treatment of a pool of porcine relaxin consisting of B29, B30, B31, and B32 relaxin led to a pure B29 relaxin after CM-cellulose chromatography at pH 7.8. During the chromatographic procedure a minor peak eluted in front of the main peak (Fig. 2), was fully active in the radioimmunoassay and migrated in the same position as B29 relaxin during cellulose acetate electrophoresis at pH 4.8. Only small differences in electrophoretic mobility were observed at pH 8.6. Since the C terminus of relaxin (B-chain) is shortened to Arg(B29) by carboxypeptidase A treatment (which leaves the A-chain unchanged), such small differences in charge could be caused only by a different amino acid at the N terminus of the A-chain or by differences in structure due to an exchange of an amino acid in B29 relaxin (the N terminus of the B-chain is blocked via a pyroglutaminyl residue).

The amino acid analysis of peak 1 (Fig. 2) showed a phenylalanine value of 1.5 residues per molecule instead of 1 for B29 relaxin. Further purification of this phenylalanine-rich material could be achieved by HPLC on reversed phase columns (Fig. 3). A fraction of relaxin-containing two phenylalanine residues could be localized in peak 5 (Fig. 3). The amino acid composition of all other amino acids was identical to B29 relaxin (Table 1) which eluted in peak 3 (Fig. 3). The more hydrophobic phenylalanine-rich relaxin was reduced and alkylated and the chains were separated via precipitation of the

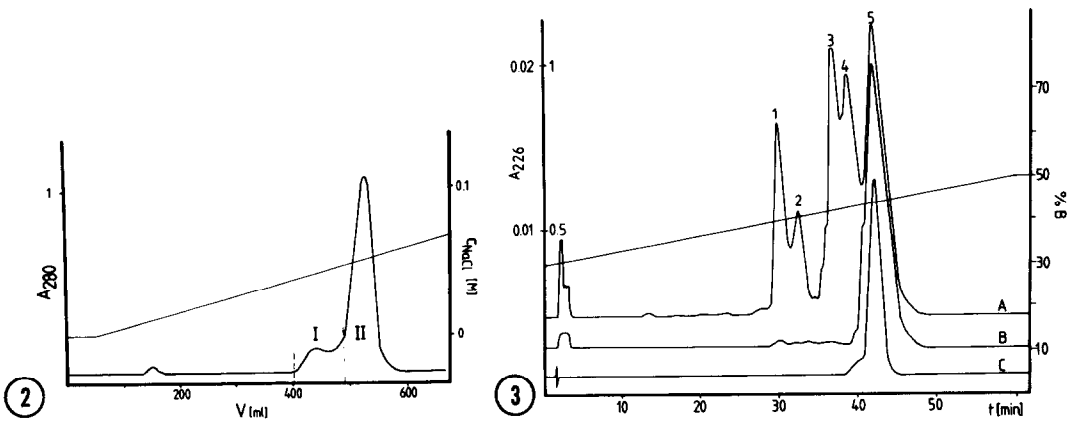

Fig. 2: CM-cellulose chromatography at pH 7.8 (7 M urea, 0.05 M tris, HCl) of 100 mg crude B29 porcine relaxin. Elution was achieved with a linear gradient 0-0.1 M NaCl (500 ml/500 ml).
Peak II: B29 relaxin.

Fig. 3: HPLC of peak I (Fig. 2) on a Synchropak RP-P column using the systems
A: 0.1% trifluoroacetic acid in water
B: 0.1% trifluoroacetic acid in 80% acetonitrile
A = preparative separation of the crude peptide mixture
B = rechromatography of peak 5
C = analytical run of rechromatographed peak 5

di(S-carboxymethyl)B-chain at pH 4 (1). Both chains were analyzed by amino acid analysis and the additional Phe was found in the A-chain (Table 1). The Phe residue was identified as N-terminal elongation of the relaxin A-chain (1,3):

```
0   1              5                  10                  15                  20    22
Phe-Arg-Met-Thr-Leu-Ser-Glu-Lys-Cys-Cys-Gln-Val-Gly-Cys-Ile-Arg-Lys-Asp-Ile-Ala-Arg-Leu-Cys
```

Table 1. Amino acid composition of [Phe^{A0}] relaxin and its chains

	[Phe^{A0}]B29 relaxin	A-chain	B-chain
Cys(Cm)	-	5.42 (4)	2.62 (2)
Asp	3.14 (3)	0.60 (1)	2.21 (2)
Thr	2.26 (2)	0.93 (1)	1.15 (1)
Ser	4.27 (4)	1.10 (1)	2.89 (3)
Glu	5.22 (5)	2.12 (2)	3.08 (3)
Gly	4.20 (4)	1.32 (1)	3.26 (3)
Ala	1.92 (2)	1.13 (1)	0.96 (1)
Cys	6.24 (6)	-	-
Val	3.93 (4)	1.03 (1)	2.69 (3)
Met	0.91 (1)	0.74 (1)	0.07 (0)
Ile	3.76 (4)	1.85 (2)	1.84 (2)
Leu	3.94 (4)	2.01 (2)	1.87 (2)
Phe	1.84 (2)	0.77 (1)	1.10 (1)
Lys	2.94 (3)	1.89 (2)	1.02 (1)
Arg	6.06 (6)	3.00 (3)	2.98 (3)
Trp^{+}	2.02 (2)	-	
fit f.$^{++}$	0.306	0.345	0.227
m.w.	6000	2700	3000

$^{+}$ Estimated by UV spectroscopy; $^{++}$the lowest fitting factor was calculated according to Alt et al. (16).

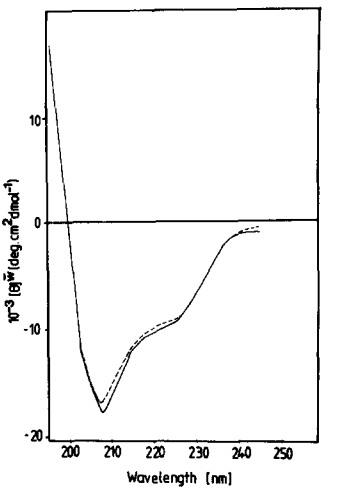

Fig. 4: Circular dichroism - spectroscopy of B29 relaxin (dashed lines) and [Phe^{A0}]B29 relaxin (solid line)

The circular dichroism spectrum of [Phe^{A0}]B29 relaxin in the near UV region at pH 7.0 has been obtained and compared to that of B29 relaxin (Fig. 4). No significant differences were detected. These data suggest that the failure to remove Phe in position A0 leaves the molecular structure essentially undisturbed.

In the radioimmunoassay, using formyltyrosyl relaxin as tracer (10), no detectable difference between B29 relaxin and [Phe^{A0}] relaxin could be observed. The mouse pubic ligament assay showed that this intermediate of relaxin biosynthesis was fully active. This is in agreement with earlier results which suggest that the preferential elongation of the N terminus of the relaxin A-chain by a formyltyrosyl residue showed no decrease in the bioactivity (10).

In HPLC of crude B29 relaxin about 10% [Phe^{A0}] relaxin are present. It cannot be isolated by the procedure of Sherwood and O'Byrne (5) via ion exchange chromatography at pH 5.5 and is therefore probably a contaminant in all of the three obtained fractions by that isolation method.

It is postulated that the [Phe^{A0}] porcine relaxin is the product of an incomplete conversion of prorelaxin to relaxin. Possibilities of the processing of porcine relaxin are discussed in detail by Haley et al. (8). The existence of [Phe^{A0}] porcine relaxin suggests that two enzymes are most likely involved in the liberation of the N terminus of the A-chain: firstly a trypsin-like enzyme which liberates [Leu-Phe^{A0}] relaxin and secondly an

aminopeptidase that causes the stepwise release of Leu and Phe. In order to prove a mechanism like this we searched for [Leu-Phe^{A0}] relaxin, but until now we have not found this intermediate in porcine relaxin preparations. Trypsin-like action by itself would cause the A-chain in rat relaxin to be 24 amino acid residues long (13,14). The cyclization to pyroglutamine probably prevented a further digest by an aminopeptidase. In the case of shark relaxin (15) the length of the A-chain is also 24 amino acid residues. However, for lack of the sequence of the connecting peptide we cannot postulate the conversion mechanism of shark relaxin.

ACKNOWLEDGEMENTS

This work was financially supported by Grants HD-10540 from HICHHD, by NSF-PGM-8302194, and supported in part by the Medical University of South Carolina Institutional Research Funds. We thank Nancy McKnight-White, Steven Martin, and Hal Crow for their excellent technical assistance.

REFERENCES

1. Schwabe, C., McDonald, J.K., and Steinetz, B.G. (1976) Biochem. Biophys. Res. Commun. 70, 397-405.
2. Schwabe, C., McDonald, J.K., and Steinetz, B.G. (1977) Biochem. Biophys. Res. Commun. 75, 503-510.
3. James, R., Niall, H., Kwok, S., and Bryant-Greenwood, G. (1977) Nature 267, 544-546.
4. Schwabe, C., and McDonald, J.K. (1977) Science 197, 914-915.
5. Sherwood, C.D., and O'Byrne, E.M. (1974) Arch. Biochem. Biophys. 160, 185-196.
6. Niall, H.D., John, M., James, R., Kwok, S., Mercado, R., Bryant-Greenwood, G., Bradshaw, R.A., Gast, M., and Boime, I. (1980) in "Insulin: Chemistry, Structure and Function of Insulin and Related Hormones" (Brandenburg, D., and Wollmer, A., eds.) pp. 719-725, de Gruyter, Berlin.
7. Walsh, J.R., and Niall, H.D. (1980) Endocrinology 107, 1258-1260.
8. Haley, J., Hudson, P., Scanlon, D., John, M., Cronk, M., Shine, J., Tregear, G., and Niall, H. (1982) DNA 1, 155-162.
9. Schwabe, C., Anastasi, A., Crow, H., McDonald, J.K., and Barrett, A.J. (1984) Biochem. J. 217, 813-817.
10. Adler, A.J., Greenfield, N.J., and Fasman, G.D. (1973) in "Methods Enzymol." 27, 675-822 (Hirs, C.H. and Timasheff, S.N., eds.) Academic Press, N.Y.
11. Schwabe, C. (1983) Endocrinology 113, 814-815.
12. Steinetz, B.G., Beach, V.L., Kroc, R.L., Stasilli, N.R., Nussbaum, R.E., Nemith, P.J., and Dun, R.K. (1960) Endocrinology 67, 102-115.
13. John, M.J., Borjesson, B.W., Walsh, J.R., and Niall, H.D. (1981) Endocrinology 108, 726-729.
14. Hudson, P., Haley, J., Cronk, M., Shine, J., and Niall, H. (1981) Nature 291, 127-131.
15. Gowan, L.K., Reinig, J.W., Schwabe, C., Bedarkar, S., and Blundell, T.L. (1981) FEBS Lett. 129, 80-82.
16. Alt, J., Heymann, E., and Krisch, K. (1975) Eur. J. Biochem. 53, 357-369.