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RELAXIN FROM AN OVIPAROUS SPECIES, THE SKATE (Raja erinacea)

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SUMMARY: An acid-acetone extract prepared from ovaries of the skate, Raja erinacea, contained a weakly crossreacting molecule when tested in a pig relaxin radioimmunoassay. The material was isolated and purified to homogeneity by ion exchange chromatography, molecular exclusion chromatography, and HPLC. Analytical tests proved the molecule to consist of two chains and to have a molecular weight of 7,500. Sequence analyses of the A and B chains yielded the following sequence: Glu-Glu-Lys-Met-Gly-Phe-Ala-Lys-Lys-Cys-Cys-Ala-Ile-Gly-Cys-Ser-Thr-Glu-Asp-Phe-Arg-Met-Val-Cys and Arg-Pro-Asn-Trp-Glu-Glu-Arg-Ser-Arg-Leu-Cys-Gly-Arg-Asp-Leu-Ile-Arg-Ala-Phe-Ile-Tyr-Leu-Cys-Gly-Gly—Thr-Arg-Trp-Thr-Arg-Leu-Pro-Asn-Phe-Gly-Asn-Tyr-Pro-Ile-Met respectively. Skate relaxin has 0.2% of the activity of B29 pig relaxin in the symphysis pubis assay and 0.5% in the mouse uterine muscle strip contraction inhibition assay.

Relaxin is responsible for the general widening of the birth canal in preparation for parturition (1) and possibly for the uterine accommodation of fetuses (2). Experimental evidence suggests that normal birth is impossible in the absence of relaxin at least in some mammalian species (3). The discovery of relaxins in viviparous sharks has led to the realization that the hormone is not limited to mammals but that it is also associated with ovoviviparity. While it has been possible to demonstrate by serological methods, i.e., crossreactivity to anti-porcine relaxin antibodies, the presence of a relaxin-like material in birds (4) and even protozoan (5), no chemical evidence has as yet been presented for the existence of this hormone in oviparous species. In this paper we are presenting the first sequence of a relaxin from the oviparous skate, Raja erinacea.

Abbreviations: PTH, phenylthiohydantoin; CM, carboxymethyl; CD, circular dichroism TFA, trifluoroacetic acid.

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MATERIALS AND METHODS: Skate ovaries (Raja erinacea) were obtained from the Mt. Desert Island Fishery Laboratory and frozen immediately after removal from the animal. The ovaries were shipped on Dry Ice. Two-kilogram batches were passed through a meat grinder without prior thawing and immediately dropped into 1 liter of cold HCl (1.6 M). The ground tissue was then homogenized with a polytron (Brinkmann) and subjected to acid/acetone fractionation according to Doczi (6). The 90% acid/acetone pellet was dissolved in water, the pH was adjusted to 5.5 using ammonium hydroxide, the solution clarified by centrifugation (5000 rpm, Sorvall RC2-B) at 4°, dialized, lyopholized and redissolved in 50 mM ammonium acetate buffer in 7 M urea at pH 5.5. The protein was applied to a CM cellulose column (2.5 x 25 cm) equilibrated with the same buffer (7) and unadsorbed protein was eluted. Thereafter a linear gradient was established with 250 ml 50 mM ammonium acetate (7 M urea) in vessel 1 and the same buffer (250 ml) containing 0.2 M in NaCl in vessel 2. The fractions were pooled according to Fig. 1 and desalted on Sephadex G-25 m in 1 M acetic acid.

Fractions 1 and 2 were further purified on a Sephadex G-50 sf column (3 x 54 cm) in 1 M acetic acid 0.15 M in NaCl; the solvent flow was adjusted to 20 ml/hr and 5 ml fractions were collected. From both fractions proteins were eluted in positions corresponding to 5000 to 8000 daltons. These were further purified by preparative reversed-phase chromatography on a Waters chromatograph and a Synchropak RP-P (C_{18}) column (250 x 10 mm). A 30% - 50% (60 min) gradient was established using 0.1% TFA in water as solvent A and 0.1% TFA in 80% acetonitrile as solvent B. The flow rate was 3 ml/min and the absorption was monitored at 226 nm.

Separation of the relaxin A and B chains was achieved by reduction and alkylation with tritiated iodoacetic acid in 6 M guanidine hydrochloride at pH 8.5 as described previously (8), followed by HPLC on an analytical Synchropak C_{18} column (250 x 4.6 mm). The chains were eluted using a 30 min gradient (20%-50% B).

The relaxin B chain was chemically cleaved at the tryptophan residues with iodosobenzoic acid according to Mahoney et al. (9). For the cleavage reaction one ml of 80% acetic acid, containing guanidine·HCl (4 M) iodosobenzoic acid (2.5 mg) and p-cresol (3.75 µl) was used to fractionate 0.5 mg of skate relaxin B chain. After 24 hr at room temperature the reaction solution was extracted with 2 x 2 ml of ether and the aqueous layer concentrated to 0.5 ml in a Speed-Vac (Savant). The peptides were separated on a Sephadex G-25 sf column (1.5 cm x 30 cm) in 1 M acetic acid. The material of interest was purified by HPLC as above using a 60 min gradient (10%-60% B). The major peak was concentrated and used for amino acid and sequence analyses.

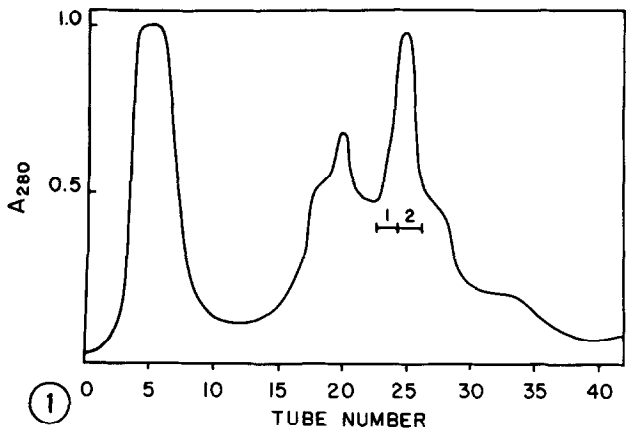
For amino acid analysis the peptides were hydrolyzed at 120° for 24 h in double-distilled bN HCl sealed in evacuated hydrolysis vials. Analyses were performed on a Dionex 502 automatic amino acid analyzer and the data obtained were used to calculate molecular weights and fit factors according to Alt et al. (10), using an Apple 2e computer.

Sequence analyses were performed on an 890C Beckman Sequencer programmed for 0.1 M quadrol. The PTH amino acids were analyzed on a DuPont HPLC system (11).

Biological activity was assessed in a mouse symphysis pubis assay (12) and the mouse uterine strip contraction inhibition assay (13).

For structural analysis in solution the CD spectrum of skate relaxin was obtained by means of a Cary 60 spectropolarimeter. The molar ellipticity was evaluated by standard methods according to Adler et al. (14).

RESULTS AND DISCUSSION: Acid-acetone extraction of 2 kg of skate ovaries yielded 1.6 g of the 90% acetone pellet. CM cellulose chromatography of the 90% acetone fraction at pH 5.5 in urea resulted in two relaxin-containing fractions of which fraction 1 contained 68 mg and fraction 2, 332 mg of protein (Figure 1). Both fractions 1 and 2 from the CM cellulose column were placed in succession onto the G-50 sf column in 1 M acetic acid 0.15 M in NaCl, and the fractions corresponding to 5000 to 8000 molecular weight were collected. After this procedure the CM 1 fraction yielded 9.3 mg of protein of the appropriate molecular weight and the CM 2 fraction 51.1 mg. Both fractions, when



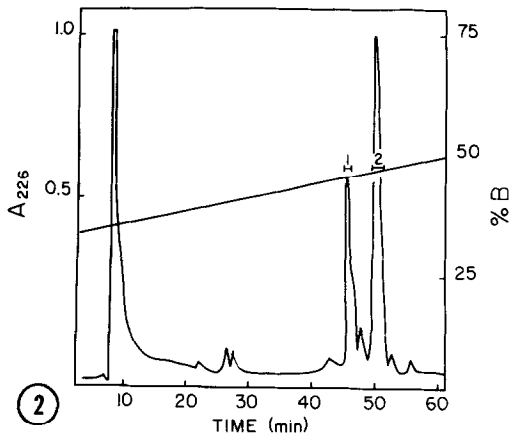


Fig. 1. Carboxymethylcellulose chromatography of a skate ovarian extract on a 2.5 x 25 cm column in 50 mM ammonium acetate, 7 M urea and a linear NaCl gradient from 0 to 0.2 M. The flow rate was 0.5 ml per minute and the fraction volume approximately 10 ml. Fractions 1 and 2 were collected.

Fig. 2. Preparative HPLC of skate relaxin fraction CM2. Fraction CM1 gave rise to an identical chromatogram except for a difference of the relative amounts of fraction 1 (relaxin I) and 2 (relaxin II), where fraction 2 contains the larger form of skate relaxin (for conditions, see Methods).

subjected to HPLC (C_{18} column), gave rise to two main peaks as shown in Figure 2. It appeared that CM1 and 2 contained the same two proteins except in different proportions. The yields of peak 1 (relaxin I) and peak 2 (relaxin II) 2 mg and 6.6 mg respectively. Relaxin II was subsequently used for all studies presented in this paper and relaxin I was characterized only by some selected procedures, like amino acid analysis, mouse uterine strip contraction inhibition assay, and CD-spectroscopy.

Upon reduction and alkylation, using tritiated iodoacetic acid of the skate relaxin, two chains could be separated by HPLC. The A chain showed peculiarly little UV absorbance but was recognized by its high radioactivity whereas the second (B chain) was retained much longer on the column and exhibited a fairly high UV absorbance (Fig. 3). The amino acid analyses of relaxin I and II and of the chains of relaxin II are shown in Table 1. Compared with relaxin II relaxin I lacks 5 amino acid residues which have been located on the C terminus of the relaxin II B-chain.

In contrast to other relaxins the relaxin of skate does not possess a pyrrolidone carboxcylic acid (PCA) residue on either chain so that sequencing could commence without prior chain fractionations. The sequence analyses of the skate relaxin A chain is summarized in Figure 4. The B chain required fractionation at the tryptophans in order to enable us to resolve unequivocally the structure of the C-terminal end of the B chain.

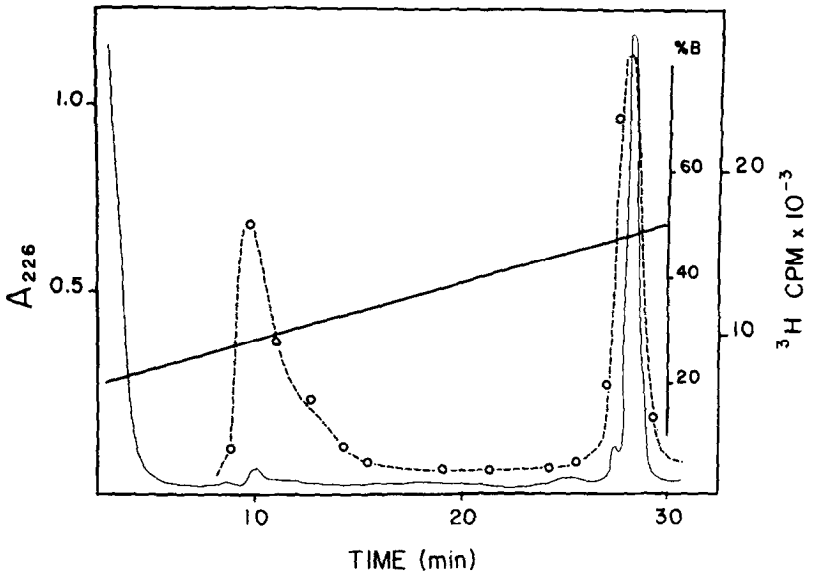


Fig. 3. Skate relaxin chain separation by HPLC. The diffuse region corresponding to the radioactivity of the ^3H (S-carboxymethyl) cysteine residues eluting after about 10 minutes contained essentially pure A chain. The hydrophobic B chain eluted in a more discrete peak about 18 minutes later (see text for details).

Table 1. Amino Acid Analyses of Skate Relaxin and Relaxin Fragments

	I	II	A-chain (II)	B Chain (II)	Ib_c peptide°(II)
Asp	3.64	4.66 (5)	1.00 (1)	3.99 (4)	1.63 (2)
Thr	2.35	2.42 (3)	0.85 (1)	1.74 (2)	0.65 (1)
Ser	1.44	1.85 (2)	0.80 (1)	1.03 (1)	0 (0)
Glu	5.02	4.99 (5)	3.16 (3)	1.87 (2)	0 (0)
Pro	2.45	4.37 (3)	0 (0)	3.23 (3)	2.04 (2)
Gly	6.01	5.88 (6)	2.08 (2)	4.00 (4)	0.86 (1)
Ala	2.89	2.89 (3)	2.10 (2)	1.03 (1)	0 (0)
Cys*	5.99	6.02 (6)	4.01 (4)+	2.06 (2)+	0 (0)
Val	1.00	1.00 (1)	0.95 (1)	0 (0)	0 (0)
Met	1.93	2.65 (3)	1.73 (2)	0.84 (1)	0** (0)
Ile	2.95	3.73 (4)	0.98 (1)	2.77 (3)	0.73 (1)
Leu	4.27	4.09 (4)	0 (0)	3.68 (4)	0.79 (0)
Tyr	0.99	2.04 (2)	0 (0)	1.81 (2)	0.99 (1)
Phe	3.95	3.99 (4)	1.95 (2)	2.13 (2)	1.03 (1)
His	0	0 (0)	0 (0)	0 (0)	0 (0)
Lys	3.04	3.33 (3)	3.08 (3)	0 (0)	0 (0)
Arg‡	8.28	8.32 (8)	1.08 (1)	7.03 (7)	1.00 (1)
Trp+	2.00	2.01 (2)	0 (0)	2.06 (2)	0 (0)
min factor	0.203	0.248	0.200	0.232	
mol weight	6800	7520	2640	4725	
mol weight (theor.)		7475	2665	4810	

* theoretical

** found as Met(0_2) but not quantified;

+ determined as carboxymethylcysteine; ° C-terminal iodosobenzoate peptide

‡ determined by UV spectroscopy

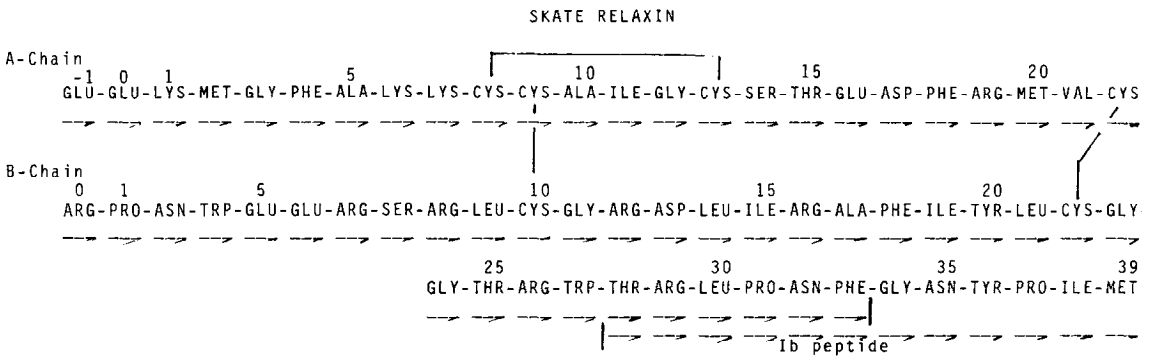


Fig. 4. The primary sequence of skate relaxin. The A chain was sequenced in one stretch with sequential yield of 90% to 98%. The cysteine positions were recognized both by PTH analysis and by radioactivity of the ^{3}H carboxymethyl cysteines. The B chain was fractionated as described in the text. The arrows designate the progression of the automatic sequence analysis and Ib designates the iodosobenzoate peptide. A five amino acid sequence overlap secures the position of the Ib peptide. (Numbering system of porcine relaxin.)

The fractionation of the B chain with iodosobenzoic acid was a fairly unsatisfactory procedure in terms of fragmentation yields (20-25%). The peptide of interest was obtained by HPLC (not shown) and was analyzed by amino acid and sequence analysis. The overlap between the two peptides was sufficient to give an unequivocal picture of the sequence of the B chain of skate relaxin (Fig. 4).

The sequence analysis of the A chain demonstrated a typical insulin/relaxin-type cysteine distribution, but remarkably one of the consistent features in all other relaxins, the two basic residues in positions 15 and 16 (pig relaxin numbering system), are occupied by threonine and glutamic acid respectively in skate relaxin. This finding not only deletes two residues from the roster of "invariant" amino acids in relaxin structures but also suggests that these two basic residues are perhaps not a part of the receptor interaction site. The A chain still contains the Gly residue in position A12 for which no structural role can be postulated from the insulin-like relaxin model (15). Other unusual features were uncovered during sequence analysis of the B chain of skate relaxin. The B chain contains two arginines in positions 12 and 16 which are identical in all relaxins, but which are not needed for the formation of the B chain alpha-helix in which they are situated. Interesting is also the presence of an Arg-Trp pair in positions 26 and 27 which is identical in all elasmobranchs, while the tryptophan in position 27 is also seen in human relaxins 1 and 2 and in pig relaxin, but not in the rat. Furthermore it is of interest to note that the tryptophan, at least in the pig relaxin, is of no functional significance (16). Skate relaxin

has the longest B chain of all relaxins so far isolated and it remains to be seen whether the length of the B chain has specific significance for the action of relaxin in the skate.

The CD spectrum of skate relaxin resembles that of dimeric insulin as concerns relative intensity of the 208/220 minima. Like pig relaxin the crossover point is at 200 nm and the 208/194 ratio is approximately 1.7 (17). These results indicate that skate relaxin has an essentially insulin-like structure in solution (Fig. 5).

The biological activity of skate relaxin in mice can be compared to that of shark relaxins (18,7). The numbers are not unequivocal but the anatomical changes occurring in the symphysis pubis make it quite clear that the mice treated with skate relaxin do show an effect upon the symphysis pubis that cannot be elicited with insulin. The relaxin appears to be recognized by the receptors but will not give rise to the same extent of symphyseal widening as does pig relaxin, for example. The same findings have been

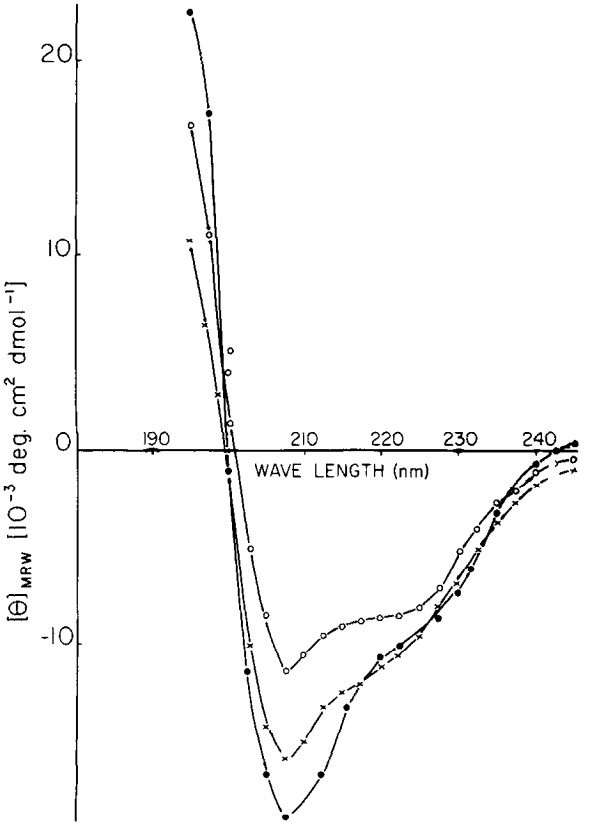


Fig. 5. CD spectra of pig (●——●). Dogfish (x——x) and skate relaxins (o——o) are displayed together for comparison. Skate relaxins I and II were indistinguishable.

Table 2. Sequence relatedness of various relaxins expressed as per cent homology

	HUMAN 1	HUMAN 2	PIG	RAT	O. TAURUS	S. ACANTHIAS	R. ERINACEA
HUMAN 1	--	77	46	46	46	45	34
HUMAN 2	77	--	46	46	48	50	35
PIG	46	46	--	54	50	52	31
RAT	46	46	54	--	37	41	25
O. TAURUS	46	48	50	37	--	75	42
S. ACANTHIAS	45	50	52	41	75	--	48
R. ERINACEA	31	35	31	25	42	48	--

reported for shark relaxins (18). Conversely the mouse uterine strip contraction inhibition assay was unequivocal if sufficient relaxin was added to the muscle bath. Pig relaxin would inhibit the spontaneous contractions of a mouse uterine muscle strip at a concentration of 2.5 ng/ml whereas an average of 500 ng/ml of skate relaxin were required to elicit the same effect. Controls such as insulin or relaxin chains were negative in the contraction inhibition assay.

The discovery of a bona fide relaxin in an oviparous animal makes for some interesting speculations both in terms of the evolution of hormonal function as well as in comparative endocrinology. It would be most interesting to know whether or not relaxin in skate has a function related to egg laying and whether relaxin is therefore a hormone of oviparity as well as viviparity.

As regards the evolution of hormonal function the skate offers some new perspectives. For a member of the Elasmobranchii one would have expected the skate to bear a close relationship to sharks. The matrix (Table 2) constructed from the known relaxin sequences shows only slightly higher homology to sand tiger shark relaxin as compared the human relaxin genes. This should be compared to the 75% homology between the two sharks, Odontaspis taurus and Squalus acanthias. Furthermore, a phylogeny constructed from relaxins would place the skate relaxin slightly closer to humans than to pig or rat which is not consistent with generally accepted views.

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