Cetacean Relaxin

ISOLATION AND SEQUENCE OF RELAXINS FROM BALAENOPTERA ACUTOROSTRATA AND BALAENOPTERA EDENI

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The tendency toward extremely high variability among relaxins derived from purportedly closely related species has come to an abrupt end with the discovery of quasi-porcine relaxin in the minke whale (Balaenoptera acutorostrata) and the Bryde’s whale (Balaenoptera edeni). An aqueous abstract of the corpora lutea of the two baleen whales contained significant amounts of relaxin-like activity as determined by a mouse bioassay and by cross-reactivity with anti-pig relaxin antibodies. The activity could be isolated and purified to homogeneity. Sequence analysis revealed that both whale relaxins differed from each other by about 3 residues, whereas the relaxin of B. edeni differed at only one position from that of pig relaxin. The similarity appears to include even the chain length heterogeneity observed at the C-terminal end of the B chain in porcine relaxin which is produced by a peculiar mode of connecting peptide removal from the prohormone. This finding may well represent one of the better documented challenges to the current paradigm of molecular evolution.

Relaxin, an insulin-like polypeptide hormone, appears to have been a crucial factor for the development of viviparity. Physiological effects attributed to relaxin include the widening of the birth canal just prior to parturition and the suppression of potentially damaging uterine contractions during gestation.

Like insulin relaxin consists of two polypeptide chains connected by two disulfide cross-links and one intrachain disulfide bond within the A chain. Although the cysteine residues are disposed as in insulin, the total homology between relaxin and insulin is less than 30%, and no biological or immunological cross-reactivity exists between the two molecules. Equally surprising is the fact that relaxins from various species show only marginal sequence relatedness although the insulin-like structure is invariant.

Relaxins isolated from the ovarian tissues of some viviparous (1, 2), oviparous (3, 4), and oviparous (5) species differ from each other by a remarkable 55–60%, whereby the apparent phylogenetic differences of the test animals are not matched by the sequence differences observed (6). In this report, we are extending our studies of relaxin structures in different species to yet another large taxon, i.e. the sea mammals.

MATERIALS AND METHODS

Ground lyophilized powders of corpora lutea were obtained from the Institute of Cetacean Research, Japan. All reagents and solvents were of analytical grade except HPLC® eluents which were HPLC grade (Burdick and Jackson, Muskegon, MI). The lyophilized luteal powder was extracted with 70% aqueous acetone (0.15 M in HCl) for about 14 h at 4°C. The extract was then centrifuged at low speed (8000 rpm) in a Sorvall RC-2B refrigerated centrifuge for 15 min at 4°C, and the supernatant was collected and brought up to 50 mM sodium acetate buffer (pH 5.5).

Ion exchange chromatography was performed on a column (1.5 x 15 cm) containing Whatman microgranular CM-cellulose equilibrated in 50 mM sodium acetate buffer, pH 5.5. Proteins adsorbed under these conditions were eluted with a linear gradient of 50 mM sodium acetate (100 ml) and 100 ml of the same buffer containing NaCl (400 mM). The radioimmunoassay-active fraction was collected as indicated in Fig. 1 and lyophilized.

The CM-cellulose fraction was then purified on a Sephadex G-50 sf column (1.5 x 30 cm) in 1 M acetic acid containing 0.15 M NaCl. Fractions eluting with an exclusion size approximately equal to porcine relaxin were collected, desalted on Sephadex G-25 in 1 M acetic acid, and further purified by HPLC.

For HPLC separation, a Waters chromatograph (pump model 6000A and solvent programmer model 660) was used in combination with a C18 column (10 x 250 mm) (Synchronap RP-3, Synchron Inc., Lindon, Utah). Absorbed material was eluted at a flow rate of 0.5 ml/min with a linear gradient of 24–40% acetonitrile containing 0.1% trifluoroacetic acid established over 30 or 60 min.

Electrophoresis was carried out in an apparatus purchased from Gelman Science (Ann Arbor, MI) with cellulose acetate strips (Sepraphore III, 2.5 x 17.1 cm). The running buffer contained urea (4 M) and was composed of 0.1 M pyridine adjusted to pH 4.8 with acetic acid. Samples were run for 2 h at 200 V and subsequently stained with 0.2% Ponceau S in 3% trichloroacetic acid.

Amino acid analysis was preceded by a vapor phase protein hydrolysis in 6 M HCl, 1% phenol under vacuum at 105°C for 24 h. Following hydrolysis, amino acids were derivatized with phenylisothiocyanate and analyzed using a Waters PicoTag system (No. 540).

The primary sequence of the A chain of both relaxins was obtained by running unmodified material (200 pmol) in an ABI 477A pulsed liquid protein sequencer and an in-line ABI 120 phenylthiohydantoin analyzer. The cysteine residues gave rise to blanks under these conditions, whereas the B chain did not degrade, indicating a blocked N-terminal amino group.

The cysteines were identified after reduction and carboxymethylation using 3H-labeled iodoacetic acid. The corresponding phenylthiohydantoin-(S-carboxymethyl)cysteine eluted in the position of the sequence observed (6).

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‡ The abbreviation used is: HPLC, high performance liquid chromatography.
phosphorylhydantoin-glutamine and was identified by its radioactivity.

Succinylation of relaxin (4 nmol) was performed in 50 µl of 0.2 M N-ethylmorpholine-HCl buffer at pH 8.5 in the presence of 100 µg of succinic anhydride (10 µl of a 10 mg/ml solution in dimethylformamide). The reaction was terminated by freezing and lyophilization after 15 min.

The B chain sequence of B. edeni relaxin was obtained after succinylation followed by incubation of 200 pmol of succinyl relaxin in 20 µl of reducing buffer (50 mM phosphate, pH 7.4, 1 mM ethylenediaminetetraacetic acid disodium salt, 0.5 mM dithio-DL-threitol) with 0.1 unit of pyroglutamyl peptidase (freshly dissolved) for 2 h at 37°C. Under these conditions, the A chain remained blocked, whereas the B chain could be sequenced without prior separation. Cysteine residues gave rise to blanks during sequence analysis under these conditions.

The B chain of the Balaenoptera acutorostrata was isolated by precipitation at pH 4.5, redissolved in reducing buffer, pH 7.4, and the N terminus was liberated using pyroglutamyl peptidase. Cysteines were identified as their carboxymethyl derivatives. Tryptic digestion of B. acutorostrata relaxin was performed with 200 µg of relaxin in 500 µl of 50 mM NH₄HCO₃ at pH 8.0 using an enzyme substrate ratio of 1:50. The digest was performed for 1 h at 37°C. Under these conditions, the enzyme substrate ratio of 1:50. The digest was performed for 1 h at 37°C. The reaction was terminated by freezing and lyophilization whereas the whole mixture was derivatized with phenylisothiocyanate for amino acid analysis.

Succinyl relaxin B-chain of B. edeni was chemically cleaved at the tryptophen residues using dimethyl sulfoxide/HCl described by Huang et al. (7).

The biological activity was measured by a mouse symphysis pubis assay (8), and immunoactivity was determined in a heterologous radioimmunoassay with sheep anti-porcine relaxin antibodies (S540) and iodinated N-formyltyrosylated porcine relaxin (9) as tracer.

RESULTS AND DISCUSSION

The strong anti-porcine relaxin antibody reaction of even a crude extract of ovaries of pregnant whales suggested that whale relaxin could be structurally closer to pig relaxin than to any of the other relaxins isolated so far (1, 3-5, 10). Relaxin-like antigenicity was indeed detected during all purification procedures, in particular chromatographic procedures, in those positions where porcine chromatography would have eluted as well.

Whale relaxins purified via CM-cellulose chromatography at pH 5.5 (Fig. 1), size separation on Sephadex G-50 sf (Fig. 2), and reversed phase HPLC (Fig. 3) were homogeneous in cellulose acetate electrophoresis at pH 4.8, migrating with an $R_v$ value identical with that of porcine relaxin. As in the case of other mammalian relaxins, UV spectroscopy indicates the presence of 2 tryptophen residues in whale relaxins. The amino acid analyses of both whale relaxins and porcine relaxin are almost identical in that proline, tyrosine, and histidine are absent. However, a significant difference between the whale relaxins and porcine relaxin is the lack of phenylalanine in the cetacean molecules (Table 1).

The sequences of the A chain of both whale relaxins were obtained by direct analysis of the unreduced molecules. The sequential yields were between 92% and 95% in both instances, a fact that we attribute to the retention of the A chains via their natural disulfide cross-links to the B chain. The absence of any signal from the B chain suggested that the N termini of the respective B chains were blocked. The cysteine residues, identified as blanks in these runs, were confirmed by separate experiments with reduced and [H]iodoacetate-labeled A chains. The assignment of the disulfide bridges was done by analogy with pig relaxin. In both cases, the sequence analysis of the A chain (Fig. 4) revealed a perfect match to the porcine hormone.

To sequence the B chains, different approaches have been taken for both molecules. The relaxin of B. acutorostrata was reduced and alkylated, and the chains were separated by precipitation of the B chain at pH 4.5. An N-terminal α-amino group was liberated using pyroglutamyl peptidase. This procedure required too much material and brought with it the disadvantage of an often incomplete pyroglutamyl peptidase removal due to the low solubility of the B chain.

In order to improve the solubility of the B chain and to
peptidase tend to break the disulfide linkages in relaxin, the pig relaxin is a leucine in both whale molecules. Although the reducing conditions required for pyroglutamyl permanently block the A chain, the relaxin of B. edeni became unrecognizable after residue Bz7 (tryptophan). In order to investigate the C-terminal region of both B chains, chain fragmentation was required.

The C-terminal region of the B chain of B. acutorostrata relaxin could be obtained after tryptic digest of the intact relaxin. The mixture was separated on Sephadex G-25 at in 50 mM ammonium bicarbonate and the C-terminal peptide identified as a tryptophan-containing peak that eluted after passage of 1 column volume of eluent. The peptide was identified by amino acid composition (Table I) and sequence analysis (Fig. 4).

The C-terminal portion of the B chain of succinylated B. edeni relaxin required chain separation after reduction and alkylation, followed by treatment with dimethyl sulfoxide/CNBr as described by Huang et al. (7) to effect cleavage at the tryptophan residues. The material was then lyophilized, redissolved, and subjected to sequence analysis. The C-terminal peptide sequence could be read together with the known sequence of the B chain fragment beginning at B19. An additional experiment with carboxypeptidase A further confirmed the C-terminal sequence of the B chain of B. edeni relaxin (Table I). While the B. edeni relaxin is identical with pig relaxin from residue B1 to the last residue (B32), the B. acutorostrata relaxin varies in 3 of the last 7 positions (see Fig. 4). The carboxypeptidase data further suggest that B31 (Ala) is the predominant form of whale relaxins followed by B29 (Leu) and only traces of B30 (Thr) (10, 11); small amounts of Gly, Ser, and Arg may be due to carboxypeptidase B contamination. This distribution of chain length, perhaps the result of a degeneracy in the prohormone conversion process, is also identical with that observed for pig relaxin. Furthermore, the biological activity of whale relaxin appears to be indistinguishable from that of pig relaxin as measured by the mouse pubic symphysis relaxation assay (8) (not shown).

The similarity between pig and whale relaxins is highly unusual although whale and land mammals appear to share similar protein structures in general. In case of relaxins, the human (12), pig (1, 2, 10), and rat (10) molecules differ from each other as much as they all differ from the relaxins of elasmobranchs (6). The whale molecule must therefore be viewed against the background of high variability among relaxins of purportedly closely related species. An evolution-

### Table I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>B. acutorostrata</th>
<th>B. edeni</th>
<th>Porcine B chain* peptide (B. acutorostrata) residues</th>
<th>B chain* C-terminal (B. edeni)</th>
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<tbody>
<tr>
<td>Aspartic acid</td>
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<td>4</td>
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<tr>
<td>Glutamic acid</td>
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</tr>
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<td>Glycine</td>
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<td>4</td>
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<td>3</td>
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<td>Histidine</td>
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<tr>
<td>Tryptophan</td>
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</table>

*Tryptic fragment.

Carboxypeptidase A digestion.

![Fig. 4](image_url)
ary tree drawn on the basis of relaxins would not be in harmony with trees obtained from other protein structures and suggests that molecular structures might not reflect genealogy in the Darwinian sense. The insulin sequences, for example, would place pigs, rats, and humans very close to each other in a phylogenetic scheme, whereas relaxin would place pig and whale very close, but whale and humans as far from each other as humans and elasmobranchs. The model of molecular evolution can not accommodate such exceptions and one must consider that recent proposals concerning the multiplicity of life's origins and the mechanism of polymerization of repetitive primordial DNA oligomers as described by Ohno (13) may be a viable alternative and that similarities and differences in primary structures of proteins may in fact be a relic of primordial chemistry (6).

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REFERENCES