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Kazuo Yamaguchi

Jennifer L. Specker
University of Rhode Island

David S. King

Yoshiharu Yokoo

Richard S. Nishioka

See next page for additional authors

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Authors

Kazuo Yamaguchi, Jennifer L. Specker, David S. King, Yoshiharu Yokoo, Richard S. Nishioka, Tetsuya Hirano, and Howard A. Bern

Complete Amino Acid Sequences of a Pair of Fish (Tilapia) Prolactins, tPRL₁₇₇ and tPRL₁₈₈*

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Kazuo Yamaguchi†‡, Jennifer L. Specker¶, David S. King||, Yoshiharu Yokoo‡, Richard S. Nishioka**, Tetsuya Hirano‡‡, and Howard A. Bern**

From the †Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Machida, Tokyo 194, Japan, the ¶Department of Zoology, University of Rhode Island, Kingston, Rhode Island 02881, the Departments of ||Genetics and **Zoology and Cancer Research Laboratory, University of California, Berkeley, California 94720, and the ‡‡Ocean Research Institute, University of Tokyo, Nakano, Tokyo 164, Japan

The complete amino acid sequences of a pair of tilapia (*Oreochromis mossambicus*) prolactins (PRLs) were determined. The larger PRL of molecular mass 20,836 Da consists of 188 amino acid residues. The smaller PRL of molecular mass 19,584 Da is 11 residues shorter. On alignment of the two sequences, the 19.6-kDa PRL (tPRL₁₇₇) has two conspicuous deletions on the NH₂-terminal side of the disulfide bond which connects the first and second cysteine residues. The degree of similarity between the two PRL sequences is unexpectedly low (130 identical residues, 69%) compared with that between the variants of other teleostean PRLs. Circular dichroism spectra and hydropathy profiles suggest structural similarity of the two PRLs. The sequence of the 20.8-kDa PRL (tPRL₁₈₈) has 69% identity with that of salmon PRL. The sequence of tPRL₁₇₇ is 56% identical with that of salmon PRL. Each tilapia PRL is equally similar to mammalian PRLs (about 30% identical residues). Regions highly conserved among teleostean and mammalian PRLs were identified on the COOH-terminal side of the disulfide bond connecting the first and second cysteine residues.

(*Cyprinus carpio*); these isohormones, in contrast with the pair of tilapia PRLs, are highly similar, differing from each other by only 4 residues and 1 residue, respectively. All fish PRLs lack 12 NH₂-terminal residues present in mammalian PRLs.

The pair of PRLs released in equal quantities from cultured tilapia pituitary glands are chemically distinct, with isoelectric points of 6.7 and 8.7 for the PRLs of mass 20 and 24 kDa, respectively, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, yet their immunogenic and physiologic properties are similar (10, 13). Both show identical activity in a bioassay designed to test for the osmoregulatory function of PRLs (14, 15); specifically, both PRLs prevented the loss of Na⁺ from hypophysectomized tilapia in fresh water (10). However, only the larger tilapia PRL promoted an increase in the length and weight of intact juvenile tilapia (13).

We now describe the determination of the complete amino acid sequences of these two tilapia PRLs. Comparison between them and other teleostean and mammalian PRLs may contribute toward understanding the relationships between structure and biological properties of PRLs and GHs.

EXPERIMENTAL PROCEDURES

Materials—Tilapia 20- and 24-kDa PRLs were purified as described by Specker *et al.* (10). The enzymes used for fragmentation were as follows: lysyl endopeptidase (Wako Pure Chemical), *Staphylococcus aureus* protease (Miles Laboratories Inc.), α -chymotrypsin (Sigma), and carboxypeptidase A (Sigma). Reagents and solvents for the gas-phase sequenator (ABI model 470A) were purchased from Applied Biosystems. Other chemicals used were of the purest grade commercially available.

Reduction and S-Carboxamidomethylation—Each tilapia PRL (100 μ g) was reduced with dithiothreitol (20 mM) in 100 μ l of 0.1 M Tris-HCl, 6 M guanidine HCl, 1 mM Na₂EDTA, pH 8.3, for 2 h at 50 °C and subsequently S-alkylated with iodoacetamide (45 mM) for 30 min at room temperature. S-Alkylated protein was desalted by reverse-phase high performance liquid chromatography (HPLC) on a YMC AM-312 ODS column (Yamamura Kagaku, 0.6 \times 15 cm, particle size 5 μ m, pore size 120 Å), eluted with a 60-min linear gradient of 0-70% aqueous acetonitrile containing 0.1% trifluoroacetic acid.

Enzymatic and Chemical Cleavage—S-Alkylated protein prepared as described above was subjected to enzymatic and chemical cleavage. For each cleavage, 2-4 nmol of protein were used. Digestion with lysyl endopeptidase was performed in 0.1 M Tris-HCl, 4 M urea, pH 9.0, at 37 °C for 4 h using 1% (w/w) enzyme. Digestion with *S. aureus* protease was performed in 0.1 M Tris-HCl, pH 7.8, at 37 °C for 24 h using 4% (w/w) enzyme. Digestion with chymotrypsin was performed in 0.1 M ammonium bicarbonate, 0.1 mM CaCl₂, pH 8.4, at 37 °C for 16 h using 1% (w/w) enzyme. Cyanogen bromide cleavage was performed with a 100 M excess of the reagent in 70% formic acid at room temperature in the dark for 24 h. The resultant peptides were separated by reverse-phase HPLC under the same conditions as described

The pituitary hormone prolactin (PRL)¹ belongs to a family of molecules that are related structurally and functionally. The family includes pituitary growth hormone (GH), placental lactogen, and placental and cell line-derived proliferin (1-5). Within mammalian species, PRLs occur in multiple molecular forms, variously called variants, isohormones, or isoforms, which differ in primary structure and/or degree of glycosylation (6-9).

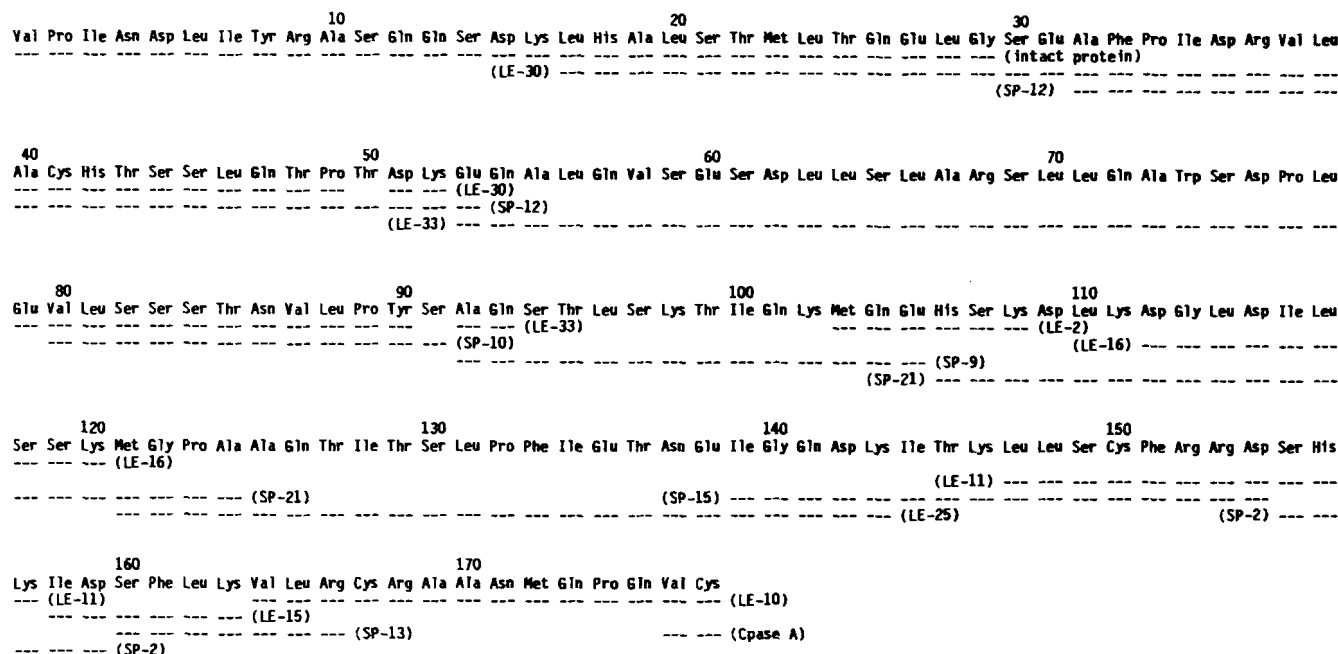
Among teleost fishes, two kinds of hormone variants have been reported. Specker *et al.* (10) reported the NH₂-terminal amino acid sequences of a pair of PRLs isolated from the tilapia (*Oreochromis mossambicus*). Within the first 26 residues there were five substitutions, suggesting moderate identity (81%). However, Yasuda *et al.* (11, 12) have reported the complete amino acid sequences of pairs of PRLs from the chum salmon (*Oncorhynchus keta*) and from the common carp

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† To whom correspondence should be addressed.

¹ The abbreviations used are: PRL, prolactin; GH, growth hormone; HPLC, high performance liquid chromatography.

A



B

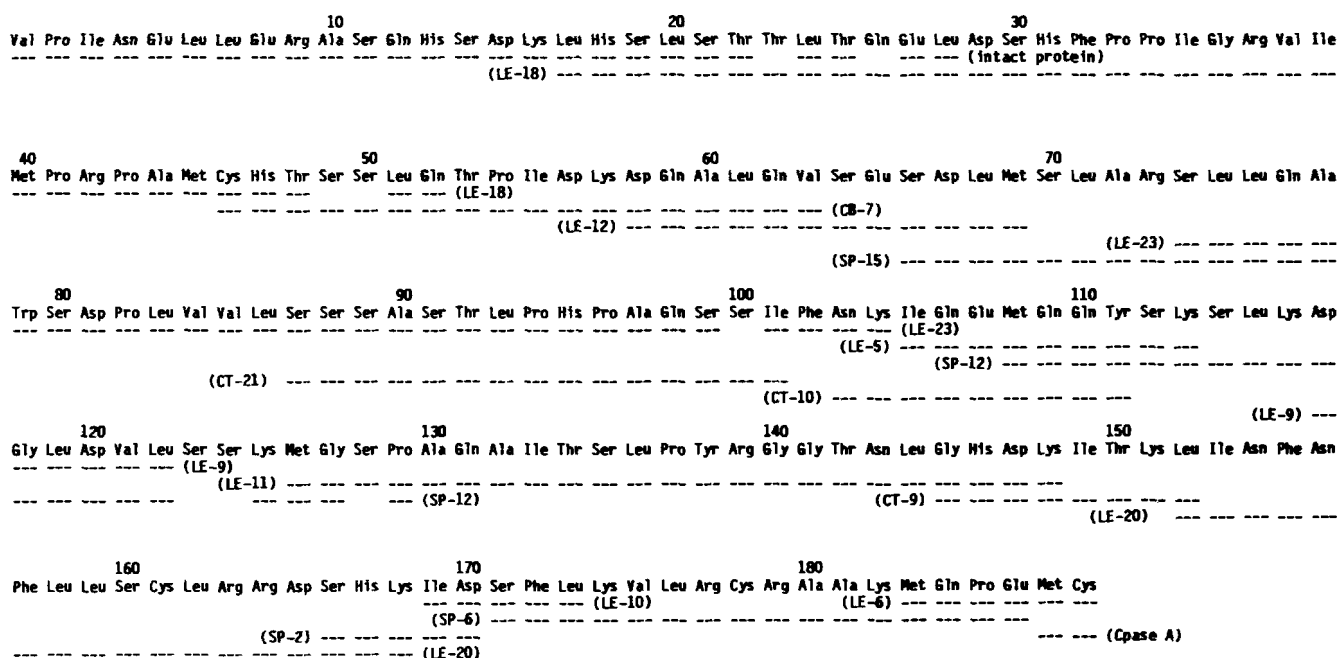


FIG. 1. A, complete amino acid sequence of 20-kDa tilapia PRL. Broken lines indicate amino acid residues determined by sequence analyses. The designations LE and SP represent peptides derived by lysyl endopeptidase and *S. aureus* protease, respectively. Cpase A represents the sequence derived by carboxypeptidase A digestion. B, complete amino acid sequence of the tilapia 24-kDa PRL. Broken lines indicate amino acid residues determined. The designations CT and CB represent peptides derived from chymotrypsin and from cyanogen bromide cleavages, respectively.

above. Carboxypeptidase digestion was performed in 0.2 M triethylamine formate, pH 8.5, at room temperature; aliquots were removed at appropriate time intervals and subjected to amino acid analysis.

Amino Acid Analysis—Two nmol of tilapia PRLs were hydrolyzed in 6 M HCl at 110 °C for 22 h. Amino acid analyses were carried out on a Jeol 200A amino acid analyzer with a standard gradient program. Amino acid analyses of the carboxypeptidase digests were performed by the Waters picoTag method of Bidlingmeyer *et al.* (16).

Sequence Analysis—Automated Edman degradation was performed

with a gas-liquid sequenator (17). Resultant phenylthiohydantoin were identified by HPLC on a C₈ reverse-phase column (Senshu Kagaku, SEQ-4, C₈, 0.46 × 30 cm, particle size 7 μm) at 40 °C using a gradient of acetonitrile in 40 mM sodium acetate buffer (pH 4.9) for elution.

Circular Dichroism Spectra—CD spectra were obtained on a Jasco 500A spectropolarimeter. Fused quartz cells of 5-mm path length were used for the measurements from 250 to 330 nm, and 0.2-mm path length for the measurements from 190 to 250 nm. Protein

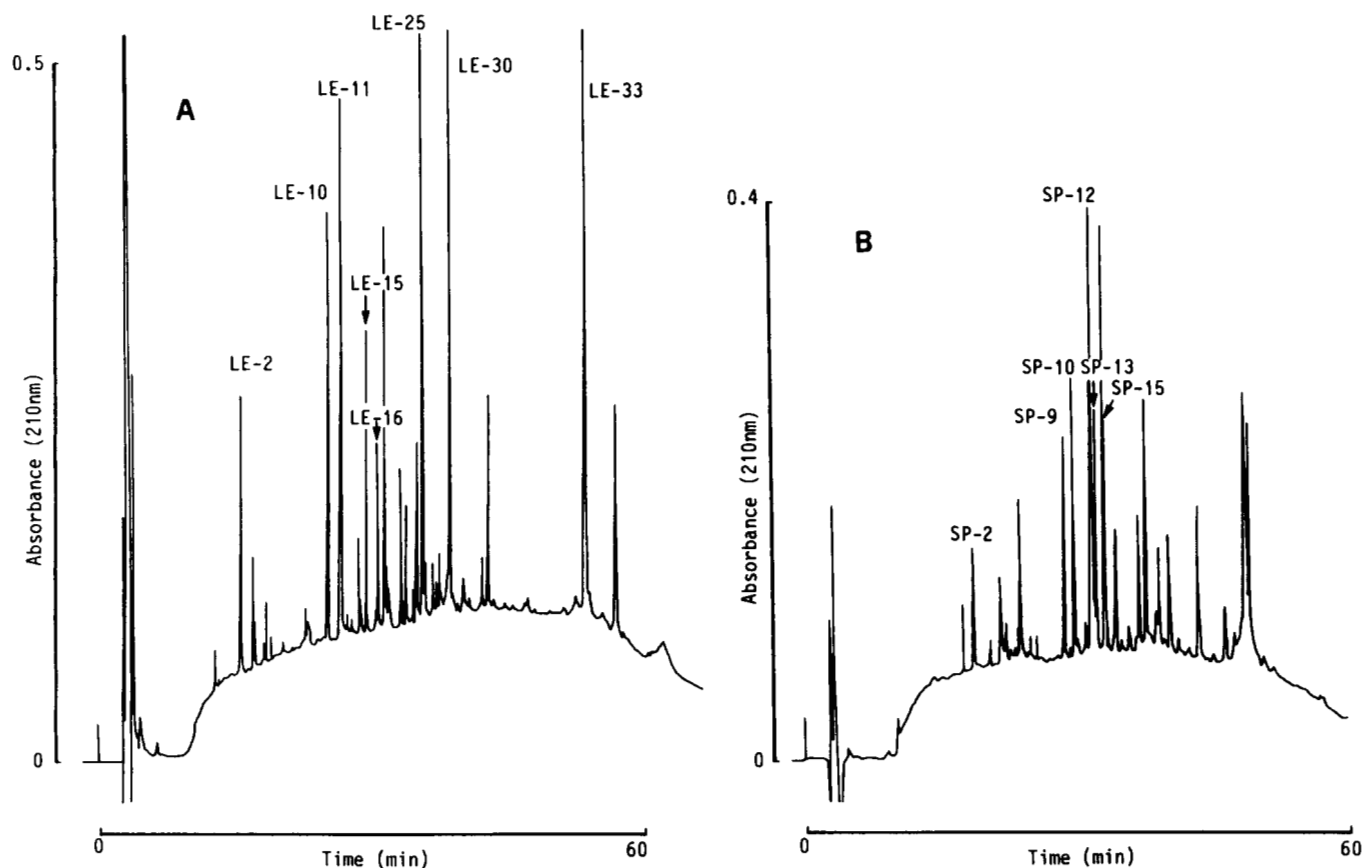


FIG. 2. A, separation by HPLC of a lysyl endopeptidase digest of 20-kDa PRL on a YMC AM-312 column (0.6 \times 15 cm, particle size 5 μ m). Elution was performed with a 60-min linear gradient of 0–70% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. B, separation by HPLC of a *S. aureus* digest of tilapia 20-kDa PRL. Chromatographic conditions are as in A.

concentrations were 0.2 mg/ml in water. Mean residue molecular ellipticities, $[\theta]_{MRW}$, were calculated using a value of 111 for the mean residue weight in both PRLs.

RESULTS

Amino Acid Sequences—Fig. 1 summarizes the amino acid sequences of the tilapia PRLs and the peptides used for sequence determination. Sequence data for the peptides are shown in Table 2.² Sequence analysis of 1 nmol of the intact *S*-carboxamidomethylated tilapia 20-kDa PRL allowed assignment of 29 NH₂-terminal residues. In order to obtain the sequences of the remaining residues, further sequence analyses of fragments from proteolytic cleavage were performed. Eight lysyl endopeptidase peptides (LE-2, LE-10, LE-11, LE-15, LE-16, LE-25, LE-30, LE-33) purified by HPLC (Fig. 2A) were analyzed for NH₂-terminal sequences. Peptide LE-30 confirmed a portion of the NH₂-terminal sequence of the intact protein and extended it through residue 52. The other lysyl endopeptidase peptides provided the determination of a total of 109 additional residues. Subsequently, seven *S. aureus* protease peptides (SP-2, SP-9, SP-10, SP-12, SP-13, SP-15, SP-21) were isolated by HPLC (Fig. 2B). They provided 17 missing residues and the alignment of lysyl endopeptidase peptides in the order LE-30, LE-33, LE-2, LE-16, LE-25, LE-11, LE-15, and LE-10. Peptide SP-10 was found to be gener-

ated by nonselective cleavage with *S. aureus* protease at the carboxyl side of Ser-91. LE-30 and LE-33 overlapped by only one glutamyl residue. However, LE-33 was the only lysyl endopeptidase peptide having an NH₂-terminal glutamic acid. In addition, the extensive sequence similarity between tilapia 20-kDa PRL and other teleostean PRLs (see below) confirmed the alignment.

Carboxypeptidase A digestion of the intact *S*-carboxamidomethyl protein provided the COOH-terminal sequence -Val-Cys (Table 3), and the complete sequence was accordingly established.

The strategy for sequence determination of 24-kDa PRL was similar to that used for 20-kDa PRL (Fig. 1 and Table 4). One nmol of the intact *S*-carboxamidomethylated 24-kDa PRL was submitted to sequence analysis, and an NH₂-terminal sequence of 26 residues was determined. Analyses of nine lysyl endopeptidase peptides (LE-5, LE-6, LE-9, LE-10, LE-11, LE-12, LE-18, LE-20, LE-23 (Fig. 3A)) and four *S. aureus* protease peptides (SP-2, SP-6, SP-12, SP-15, Fig. 3B) provided the sequences of a total of 143 residues. Nine hitherto unidentified residues and two overlaps were established by analyses of peptides CT-9, CT-10, CT-21, and CB-7 obtained by chymotryptic digestion and cyanogen bromide cleavage (Fig. 3, C and D). CT-9 overlaps LE-20 by only 1 residue, Leu-151. However, LE-20 was the only lysyl endopeptidase peptide having an NH₂-terminal leucine with the exception of LE-18, which had been aligned at 17–57. The considerable sequence similarity between tilapia 24-kDa PRL and tilapia 20-kDa PRL (see below) confirmed the alignment. Carboxypeptidase A digestion of intact *S*-carboxamidomethylated protein provided the COOH-terminal sequence -Met-Cys (Table

² Tables 2–5 are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

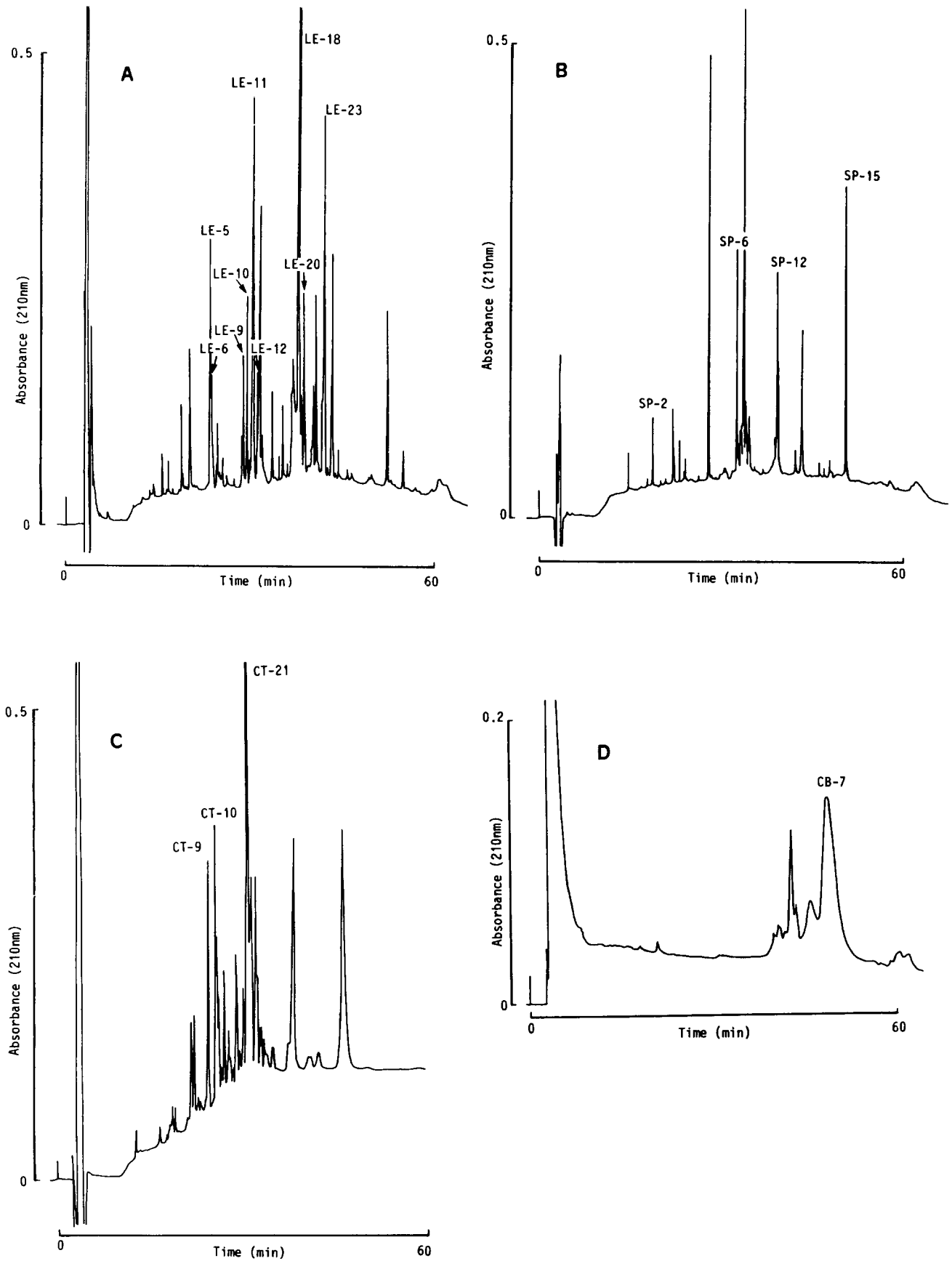


FIG. 3. A, separation by HPLC of a lysyl endopeptidase digest of tilapia 24-kDa PRL. Chromatographic conditions are as in Fig. 2A. B, separation by HPLC of a *S. aureus* protease digest of tilapia 24-kDa PRL. Chromatographic conditions are as in Fig. 2. C, separation by HPLC of a chymotrypsin digest of tilapia 24-kDa PRL. Chromatographic conditions are as in Fig. 2. D, separation by HPLC of the peptides generated by cyanogen bromide cleavage of 24-kDa PRL. Chromatographic conditions are as in Fig. 2.

5). Thus, the complete amino acid sequence of 24-kDa PRL was established.

The amino acid compositions of the two PRLs, shown in Table 1, are in agreement with the results of amino acid sequence determinations. Molecular masses were calculated to be 19,584 Da for 20-kDa PRL and 20,836 Da for 24-kDa PRL. The mass calculated for the 24-kDa PRL is lower than that estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10). Sequence analyses confirmed that it has no glycosylation site such as is present in mammalian PRLs (7).

CD Spectra—The CD spectra of the two PRLs are shown in Fig. 4. The two spectra are similar in the region of amide bond absorption (Fig. 4A). The position of two negative bands

at 209 nm and 221 nm in this region is typical of α -helical polypeptides. The relative intensities of these two bands are similar to those of human GH reported by Bewley *et al.* (18). The α -helix content estimated by the method of Bewley and Li (19) is about 45% in each of the PRLs. The CD spectrum

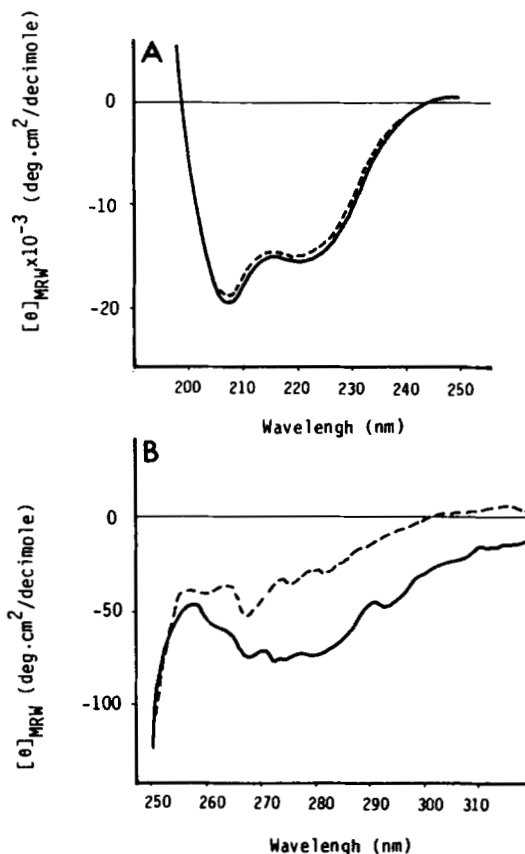


FIG. 4. Circular dichroism spectra of 20-kDa PRL (—) and the 24-kDa PRL (---) in H₂O. A, amide bond circular dichroism spectra. B, side-chain circular dichroism spectra.

TABLE I
Amino acid composition of 20- and 24-kDa PRL

| Residue | 20 kDa | 24 kDa |
|---------|-------------------------------------|-------------|
| Asx | 16.9 ^a (16) ^b | 16.9 (16) |
| Thr | 11.9 (12) | 10.2 (9) |
| Ser | 20.8 (24) | 23.1 (27) |
| Glx | 21.3 (22) | 18.6 (18) |
| Pro | 7.9 (8) | 12.2 (12) |
| Gly | 5.0 (4) | 6.9 (6) |
| Ala | 13.1 (12) | 11.7 (11) |
| ½Cys | 3.5 (4) | 3.6 (4) |
| Val | 6.7 (7) | 6.0 (7) |
| Met | 3.7 (4) | 6.6 (7) |
| Ile | 9.8 (10) | 9.9 (10) |
| Leu | 26.6 (25) | 27.1 (26) |
| Tyr | 1.1 (2) | 1.6 (2) |
| Phe | 4.5 (4) | 4.9 (5) |
| His | 4.7 (4) | 7.3 (7) |
| Lys | 11.6 (11) | 11.4 (11) |
| Arg | 7.2 (7) | 9.1 (9) |
| Trp | ND ^c (1) | ND (1) |
| Total | 176.3 (177) | 187.1 (188) |

^a Values indicate the number of residues/molecule.
^b Numbers in parentheses represent the number of residues determined by sequence analysis.
^c ND, not determined.

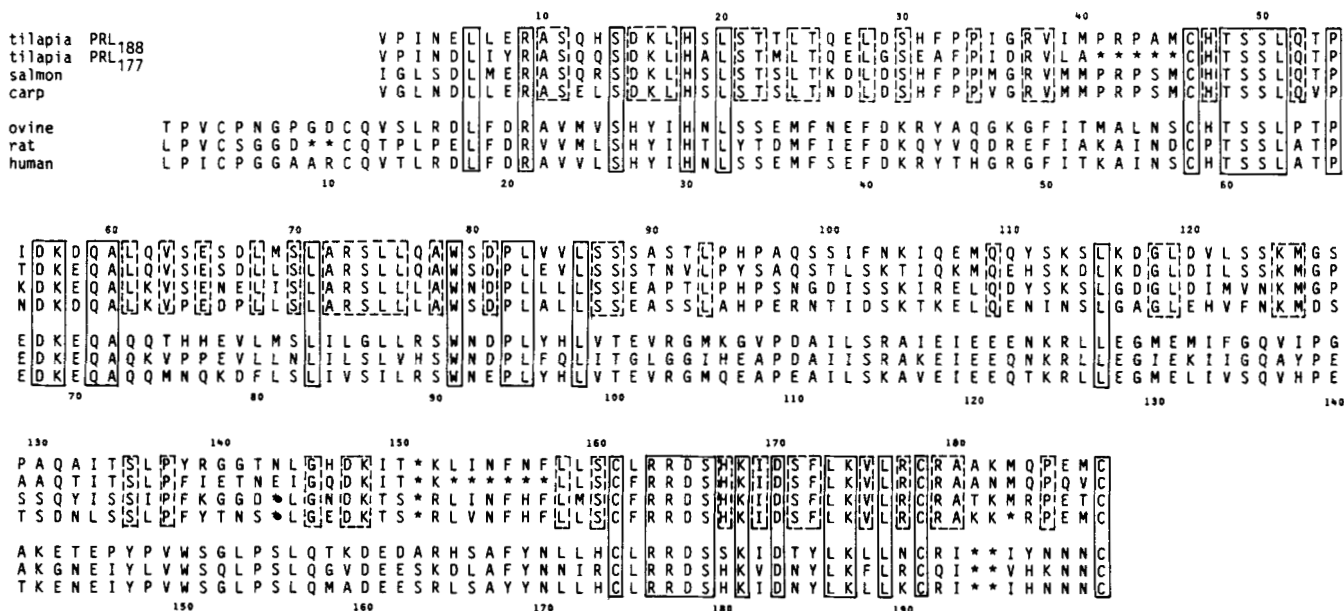


FIG. 5. Alignment of amino acid sequences (represented by standard single-letter abbreviations) of tPRL₁₇₇ and tPRL₁₈₈ with those of PRLs from salmon (11), carp (12), sheep (20), rat (21), and human (22). Identical residues among the teleost PRLs are boxed with a broken line. Identical residues in teleost and mammal PRLs are boxed with a solid line.

in the region of aromatic side chain absorption shows some differences; neither spectrum shows a positive tryptophan band above 290 nm, which was observed in ovine PRL and human GH (18). At the present time we have no interpretation to offer for these findings.

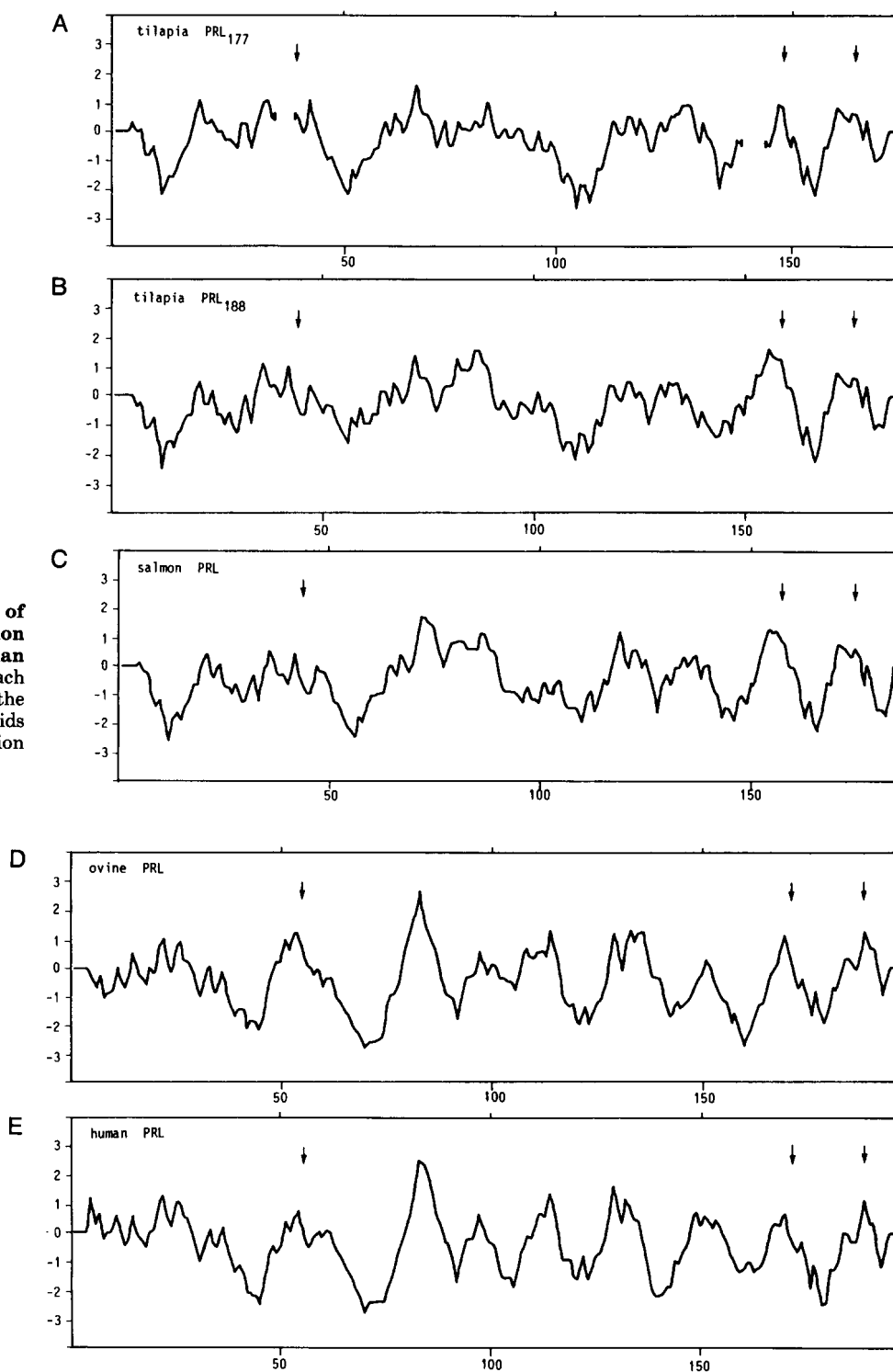
DISCUSSION

The complete amino acid sequences of the two tilapia PRLs surprisingly disclose much less similarity than exists between the two salmon PRLs and between the two carp PRLs. The sequence identity for the entire molecule is only 69% (130/188), considerably less than that found in the NH₂-terminal

sequence (81%, 21/26) (10). The larger tilapia PRL, previously termed 24-kDa PRL and henceforth termed tPRL₁₈₈, contains 188 residues. The smaller tilapia PRL, previously termed 20-kDa PRL and henceforth called tPRL₁₇₇, contains 177 residues. The more basic isoelectric point of tPRL₁₈₈ (8.7) compared with that of tPRL₁₇₇ (6.7) (10) can now be explained by the larger number of basic residues and the smaller number of acidic residues.

Fig. 5 details the complete amino acid sequences of the two tilapia PRLs, along with those of some other teleostean and mammalian PRLs. Sequences have been aligned to optimize similarity. The teleostean PRLs all lack the first 12 amino

FIG. 6. Hydropathy profiles of tPRL₁₇₇ (A), tPRL₁₈₈ (B), salmon PRL (C), ovine PRL (D), and human PRL (E). A hydropathy score for each residue was obtained by averaging the hydropathy indices (34) of 9 amino acids at a time. Arrows indicate the position of cysteine residues.



acid residues present in mammalian PRLs and, consequently, the first disulfide loop that exists in mammalian PRLs. In having similar numbers of residues and four conserved cysteine residues rather than six, they more closely resemble GHs. Each teleostean PRL is only about 30% identical to the mammalian PRLs. tPRL₁₇₇ is about 56% (105/188) identical to salmon PRL and 51% (96/188) identical to carp PRL. tPRL₁₈₈ is 69% (129/188) identical to salmon PRL and 64% (121/188) identical to carp PRL.

The degree of identity between the two tilapia PRLs (69%) is no higher than that between tilapia PRLs and the other teleostean PRLs. Compared to tPRL₁₈₈, tPRL₁₇₇ lacks two short sequences: 5 residues (41–45) and 6 residues (152–157) preceding the first cysteines of the two disulfide bonds which are likely to be present (see below). The 58 substitutions found between the two tilapia PRLs are distributed along the entire molecule. This extends our earlier contention that these two forms are distinct at the pretranslational level (10, 13) and are probably products of two separate genes. Further, the greater similarity of the larger than the smaller tilapia PRL to the other teleostean PRLs (69 and 64% compared with 56 and 51%) suggests that receptor systems and bioassay systems might distinguish tPRL₁₇₇ from other teleostean PRLs. In this regard, it is interesting that in intact juvenile tilapia, ovine PRL, bovine GH, and the larger tilapia PRL were found to stimulate growth, whereas neither tilapia GH nor the smaller tilapia PRL had such an effect (13).

In an amphibian bioassay system, tPRL₁₇₇, but not tPRL₁₈₈ nor various GHs, behaves similarly to ovine and amphibian PRL.³ We might speculate that overall these findings suggest that conservative evolutionary pressure on the tilapia PRLs remained on the larger, leaving the smaller one free to change and take on other functions or to lose original functions. Specker *et al.* (23) proposed that the PRL cell is a renegade growth hormone cell. We can now propose that tPRL₁₇₇ is a renegade PRL molecule which has lost growth hormone-like characteristics.

Identical residues among the teleostean and the mammalian PRLs (see Fig. 5) are clustered in four highly conserved regions located at the alignment positions 6–20, 46–60, 71–86, and 161–178. Residues involved in receptor binding are thought to be located within these highly conserved regions, since ovine PRL shows high binding affinity to the tilapia PRL receptor (24), and conversely, PRLs of some teleosts have low but significant activity in mammalian PRL assays (25–27). Recently, Nicoll *et al.* (4) compared the amino acid sequences of mammalian GHs and PRLs in an attempt to identify the regions involved in receptor binding and in ensuring hormone specificity and generating species specificity. They suggested that four clusters of residues are the determinants for receptor binding corresponding to positions 3–8, 50–57, 78–82, and 127–136 of the teleostean PRL sequences. Among these, only the first three clusters correspond with regions highly conserved between teleostean and mammalian PRL sequences and are thus likely to contain determinants for receptor binding of teleostean PRL.

Because the 1–134 segment of human GH has binding affinity for both mammalian PRL and GH receptors (28, 29), the NH₂-terminal two-thirds of PRL and GH are thought to be important for receptor binding. However, the 1–134 fragment has low potency *in vivo* (30). In contrast, two-chain forms of human GH obtained by enzymatic cleavage in the region between residues 134 and 150 (31, 32) and two-chain forms of rat PRL cleaved at about the same position (33) retain full activity in *in vivo* mammalian assays. These results

suggest that COOH-terminal portions of the molecules ensure hormone specific activity *in vivo*. Teleostean and mammalian PRLs are thought to have similar tertiary structures as judged from sequence similarity, similar circular dichroism spectra in the region of amide bond absorption, and similar hydrophathy profiles (Fig. 6). Therefore, teleostean PRLs probably have two disulfide bonds formed by cysteines 46–161 and cysteines 178–188, corresponding to the similarly located disulfide bonds present in mammalian PRLs (20). The closest similarity between the PRLs is on the COOH-terminal side of cysteine 161; another highly similar region is on the COOH-terminal side of cysteine 46. These highly conserved regions are thought to be exposed to the outside of the molecule as indicated by low hydrophathy values (Fig. 6) and are probably essential for biological activity of mammalian PRLs as well as teleostean PRLs.

The new information on the structures of this pair of PRLs supports our earlier conclusion, based more on biological and immunological information (10, 13), that the tilapia PRLs are importantly similar, and possibly importantly different, in their tertiary structures. Thus, one conclusion, possibly important to efforts to modify PRL or GH functions using recombinant DNA techniques, is that two deletions from tPRL₁₇₇, both located on the NH₂-terminal side of disulfide bonds, are of no consequence to the tertiary structure which ensures PRL's osmoregulatory function; however, their loss, and/or alteration in residues, seems to have led to decreased effectiveness in promoting juvenile growth.

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Supplementary Material to
Complete Amino Acid Sequences of a Pair of Fish(Tilapia)
Prolactins.

by
Kazuo Yamaguchi, Jennifer L. Specker, David S. King,
Yoshiharu Yokoo, Richard S. Nishioka, Tetsuya Hirano, and
Howard A. Bern

Table 2. Amino acid sequence data for carboxamidomethylated
[PRL177]

| Position | Residue | Peptide, (cycle), yield, (pmole) |
|----------|---------|----------------------------------|
| | | <u>Intact Protein</u> |
| 1 | Val | (1) 770 |
| 2 | Pro | (2) 847 |
| 3 | Ile | (3) 834 |
| 4 | Asn | (4) 704 |
| 5 | Asp | (5) 500 |
| 6 | Leu | (6) 910 |
| 7 | Ile | (7) 921 |
| 8 | Tyr | (8) 728 |
| 9 | Arg | (9) 668 |
| 10 | Ala | (10) 860 |
| 11 | Ser | (11) 150 |
| 12 | Gln | (12) 510 |
| 13 | Gln | (13) 675 |
| 14 | Ser | (14) 120 |
| 15 | Asp | (15) 175 |
| 16 | Lys | (16) 460 |
| 17 | Leu | (17) 532 |
| 18 | His | (18) 175 |
| 19 | Ala | (19) 326 |
| 20 | Leu | (20) 480 |
| 21 | Ser | (21) 112 |
| 22 | Thr | (22) 52 |
| 23 | Met | (23) 195 |
| 24 | Leu | (24) 440 |
| 25 | Thr | (25) 66 |
| 26 | Gln | (26) 198 |
| 27 | Glu | (27) 120 |
| 28 | Leu | (28) 310 |
| 29 | Gly | (29) 86 |
| 30 | Ser | (30) 68 |
| 31 | Glu | (31) 122 |
| 32 | Ala | (32) 1075 |
| 33 | Phe | (33) 178 |
| 34 | Pro | (34) 102 |
| 35 | Ile | (35) 146 |
| 36 | Asp | (36) 70 |
| 37 | Arg | (37) 48 |
| 38 | Val | (38) 113 |
| 39 | Leu | (39) 102 |
| 40 | Ala | (40) 101 |
| 41 | Cys | (41) 25 |
| 42 | His | (42) 15 |
| 43 | Thr | (43) 10 |
| 44 | Ser | (44) 42 |
| 45 | Ser | (45) 46 |
| 46 | Leu | (46) 41 |
| 47 | Gln | (47) 14 |
| 48 | Thr | (48) 5 |
| 49 | Pro | (49) 13 |
| 50 | Thr | (50) 34 |
| 51 | Asp | (51) 5 |
| 52 | Lys | (52) 10 |
| 53 | Glu | (53) 434 |
| 54 | Gln | (54) 888 |
| 55 | Ala | (55) 863 |
| 56 | Leu | (56) 905 |
| 57 | Gln | (57) 717 |
| 58 | Val | (58) 715 |
| 59 | Ser | (59) 310 |
| 60 | Glu | (60) 165 |
| 61 | Ser | (61) 256 |
| 62 | Asp | (62) 160 |
| 63 | Leu | (63) 372 |
| 64 | Leu | (64) 412 |
| 65 | Ser | (65) 160 |
| 66 | Leu | (66) 291 |
| 67 | Ala | (67) 210 |
| 68 | Arg | (68) 45 |
| 69 | Ser | (69) 176 |
| 70 | Leu | (70) 215 |
| 71 | Leu | (71) 192 |
| 72 | Gln | (72) 165 |
| 73 | Ala | (73) 142 |
| 74 | Trp | (74) 65 |
| 75 | Ser | (75) 105 |
| 76 | Asp | (76) 36 |
| 77 | Pro | (77) 54 |

| | | | |
|-----|-----|--------------|--------------|
| 78 | Leu | (26) 102 | <u>SP-10</u> |
| 79 | Glu | (27) 28 | |
| 80 | Val | (28) 72 | (1) 636 |
| 81 | Leu | (29) 100 | (2) 539 |
| 82 | Ser | (30) 72 | (3) 240 |
| 83 | Ser | (31) 85 | (4) 240 |
| 84 | Ser | (32) 76 | (5) 202 |
| 85 | Thr | (33) 6 | (6) 43 |
| 86 | Asn | (34) 18 | (7) 211 |
| 87 | Val | (35) 30 | (8) 270 |
| 88 | Leu | (36) 74 | (9) 221 |
| 89 | Pro | (37) 22 | (10) 145 |
| 90 | Tyr | (38) 24 | (11) 192 |
| 91 | Ser | (39) - | (12) 35 |
| 92 | Ala | (30) 22 | (1) 635 |
| 93 | Gln | (41) 27 | (2) 450 |
| 94 | Ser | | (3) 265 |
| 95 | Thr | | (4) 66 |
| 96 | Leu | | (5) 150 |
| 97 | Ser | | (6) 126 |
| 98 | Lys | | (7) 63 |
| 99 | Thr | | (8) 25 |
| 100 | Ile | | (9) 265 |
| 101 | Gln | <u>LE-2</u> | (10) 178 |
| 102 | Lys | | (11) 60 |
| 103 | Met | (1) 845 | (12) 118 |
| 104 | Gln | (2) 860 | (13) 56 |
| 105 | Glu | (3) 685 | (14) 19 |
| 106 | His | (4) 103 | (1) 102 |
| 107 | Ser | (5) 454 | (2) 40 |
| 108 | Lys | (6) 295 | (3) 65 |
| 109 | Asp | | (4) 46 |
| 110 | Leu | <u>LE-16</u> | (5) 120 |
| 111 | Lys | | (6) 52 |
| 112 | Asp | (1) 160 | (7) 39 |
| 113 | Gly | (2) 164 | (8) 58 |
| 114 | Leu | (3) 215 | (9) 90 |
| 115 | Asp | (4) 61 | (10) 28 |
| 116 | Ile | (5) 200 | (11) 50 |
| 117 | Leu | (6) 190 | (12) 50 |
| 118 | Ser | (7) 72 | (13) 20 |
| 119 | Ser | (8) 70 | (14) 20 |
| 120 | Lys | (9) 24 | (15) 10 |
| 121 | Met | (16) 14 | (1) 522 |
| 122 | Gly | (17) 19 | (2) 392 |
| 123 | Pro | (18) 8 | (3) 445 |
| 124 | Ala | (19) 16 | (4) 680 |
| 125 | Ala | | (5) 590 |
| 126 | Gln | | (6) 488 |
| 127 | Thr | | (7) 118 |
| 128 | Ile | | (8) 536 |
| 129 | Thr | | (9) 78 |
| 130 | Ser | | (10) 140 |
| 131 | Leu | | (11) 305 |
| 132 | Pro | | (12) 180 |
| 133 | Phe | | (13) 230 |
| 134 | Ile | | (14) 85 |
| 135 | Glu | | (15) 36 |
| 136 | Thr | | (16) 30 |
| 137 | Asn | | (17) 74 |
| 138 | Glu | <u>SP-15</u> | (18) 50 |
| 139 | Ile | (1) 288 | (19) 152 |
| 140 | Gly | (2) 279 | (20) 102 |
| 141 | Gln | (3) 292 | (21) 84 |
| 142 | Asp | (4) 72 | (22) 30 |
| 143 | Lys | (5) 120 | (23) 8 |
| 144 | Ile | (6) 200 | |
| 145 | Thr | (7) 54 | <u>LE-11</u> |
| 146 | Lys | (8) 106 | |
| 147 | Leu | (9) 228 | (1) 1042 |
| 148 | Leu | (10) 222 | (2) 912 |
| 149 | Ser | (11) 68 | (3) 292 |
| 150 | Cys | (12) 46 | (4) 538 |
| 151 | Phe | (13) 132 | (5) 655 |
| 152 | Arg | (14) 24 | (6) 81 |
| 153 | Arg | (15) 20 | (7) 105 |
| 154 | Asp | (16) 6 | (8) 122 |
| 155 | Ser | | (9) 168 |
| 156 | His | | (10) 30 |
| 157 | Lys | | (11) 32 |
| 158 | Ile | (1) 1100 | <u>SP-13</u> |
| 159 | Asp | (2) 624 | (4) 210 |
| 160 | Ser | (3) 294 | (5) 36 |
| 161 | Phe | (4) 745 | |
| 162 | Leu | (5) 672 | |
| 163 | Lys | (6) 304 | <u>LE-10</u> |
| 164 | Val | | (6) 352 |
| 165 | Leu | | (7) 1080 |
| 166 | Arg | | (8) 80 |
| 167 | Cys | | (9) 442 |
| 168 | Arg | | (10) 106 |
| 169 | Ala | | (11) 434 |
| 170 | Ala | | (12) 392 |
| 171 | Asn | | (13) 208 |
| 172 | Met | | (14) 181 |
| 173 | Gln | | (15) 145 |
| 174 | Pro | | (16) 142 |
| 175 | Gln | | (17) 172 |
| 176 | Val | | (18) 43 |
| 177 | Cys | | (19) 8 |

Table 3. Carboxypeptidase A digestion of carboxamidomethylated tPR1188

| Time (min) | Residues/mole | |
|------------|---------------|---------------------------------|
| | Val(176) | Carboxamidomethyl-cysteine(177) |
| 10 | 0.21 | 0.37 |
| 30 | 0.82 | 0.87 |
| 120 | 0.92 | 1.0 |

Table 4. Amino acid sequence data for carboxamidomethylated tPR1188

| Position | Residue | Peptide, (cycle), yield, (pmole) |
|-----------------------|---------|----------------------------------|
| <u>Intact Protein</u> | | |
| 1 | Val | (1) 771 |
| 2 | Pro | (2) 383 |
| 3 | Ile | (3) 691 |
| 4 | Asn | (4) 435 |
| 5 | Glu | (5) 373 |
| 6 | Leu | (6) 418 |
| 7 | Leu | (7) 622 |
| 8 | Glu | (8) 298 |
| 9 | Arg | (9) 52 |
| 10 | Ala | (10) 383 |
| 11 | Ser | (11) 72 |
| 12 | Gln | (12) 243 |
| 13 | His | (13) 43 |
| 14 | Ser | (14) 62 |
| 15 | Asp | (15) 73 |
| 16 | Lys | (16) 197 |
| 17 | Leu | (17) 188 |
| 18 | His | (18) 20 |
| 19 | Ser | (19) 42 |
| 20 | Leu | (20) 166 |
| 21 | Ser | (21) 33 |
| 22 | Thr | (22) 23 |
| 23 | Thr | (23) - |
| 24 | Leu | (24) 115 |
| 25 | Thr | (25) 10 |
| 26 | Gln | (26) - |
| 27 | Glu | (27) 60 |
| 28 | Leu | (28) 110 |
| 29 | Asp | (29) 143 |
| 30 | Ser | (30) 58 |
| 31 | His | (31) 40 |
| 32 | Phe | (32) 107 |
| 33 | Pro | (33) 110 |
| 34 | Pro | (34) 170 |
| 35 | Ile | (35) 313 |
| 36 | Gly | (36) 77 |
| 37 | Arg | (37) 41 |
| 38 | Val | (38) 87 |
| 39 | Ile | (39) 112 |
| 40 | Met | (40) 60 |
| 41 | Pro | (41) 28 |
| 42 | Arg | (42) 28 |
| 43 | Pro | (43) 90 |
| 44 | Ala | (44) 30 |
| 45 | Met | (45) 25 |
| 46 | Cys | (46) 17 |
| 47 | His | (47) 9 |
| 48 | Thr | (48) 7 |
| 49 | Ser | (49) - |
| 50 | Ser | (50) - |
| 51 | Leu | (51) 14 |
| 52 | Gln | (52) 11 |
| 53 | Thr | (53) 16 |
| 54 | Pro | (54) 76 |
| 55 | Ile | (55) 71 |
| 56 | Asp | (56) 80 |
| 57 | Lys | (57) 59 |
| 58 | Asp | (58) 36 |
| 59 | Gln | (59) 56 |
| 60 | Ala | (60) 45 |
| 61 | Leu | (61) 66 |
| 62 | Gln | (62) 33 |
| 63 | Val | (63) 44 |
| 64 | Ser | (64) 15 |
| 65 | Glu | (65) 23 |
| 66 | Ser | (66) 16 |
| 67 | Asp | (67) 18 |
| 68 | Leu | (68) 24 |
| 69 | Met | (69) 6 |
| 70 | Ser | (70) 35 |
| 71 | Leu | (71) 79 |
| 72 | Ala | (72) 86 |
| 73 | Arg | (73) 22 |
| 74 | Ser | (74) 186 |
| 75 | Leu | (75) 431 |
| 76 | Leu | (76) 430 |
| 77 | Gln | (77) 365 |
| 78 | Ala | (78) 258 |
| 79 | T p | (79) 58 |
| 80 | Ser | (80) 63 |
| 81 | Asp | (81) 202 |
| 82 | Pro | (82) 175 |
| 83 | Leu | (83) 160 |
| 84 | Val | (84) 128 |
| 85 | Val | (85) 160 |
| 86 | Leu | (86) 105 |
| 87 | Ser | (87) 56 |
| 88 | Ser | (88) 58 |
| 89 | Ser | (89) 47 |
| 90 | Ala | (90) 51 |

| | | | |
|-----|-----|------------|---------------|
| 91 | Ser | (91) 25 | (5) 117 |
| 92 | Thr | (92) 10 | (6) 38 |
| 93 | Leu | (93) 26 | (7) 218 |
| 94 | Pro | (94) 35 | (8) 216 |
| 95 | His | (95) 9 | (9) 114 |
| 96 | Pro | (96) 24 | (10) 178 |
| 97 | Ala | (97) 11 | (11) 145 |
| 98 | Gln | (98) 16 | (12) 198 |
| 99 | Ser | (99) 13 | (13) 70 |
| 100 | Ser | (100) - | (14) 41 |
| 101 | Ile | (101) 11 | (15) 10 |
| 102 | Phe | (102) 11 | |
| 103 | Asn | (103) 6 | LE-5 (1) 762 |
| 104 | Lys | (104) 6 | (2) 556 |
| 105 | Ile | (105) - | (3) 671 |
| 106 | Gln | (106) - | (4) 578 |
| 107 | Glu | (107) - | (5) 560 |
| 108 | Met | (108) 60 | (6) 630 |
| 109 | Gln | (109) 62 | (7) 381 |
| 110 | Gln | (110) 54 | (8) 380 |
| 111 | Tyr | (111) 34 | (9) 179 |
| 112 | Ser | (112) 20 | (8) 91 |
| 113 | Lys | (113) 47 | (9) 58 |
| 114 | Ser | (114) 19 | |
| 115 | Leu | (115) 45 | LE-9 (1) 410 |
| 116 | Lys | (116) 52 | (2) 420 |
| 117 | Asp | (117) 24 | (3) 520 |
| 118 | Gly | (118) 18 | (4) 388 |
| 119 | Leu | (119) 40 | (5) 385 |
| 120 | Asp | (120) 41 | (6) 447 |
| 121 | Val | (121) 33 | (7) 177 |
| 122 | Leu | (122) 28 | (8) 186 |
| 123 | Ser | (123) - | (9) 40 |
| 124 | Ser | (124) - | (7) 45 |
| 125 | Lys | (125) 16 | LE-17 (1) 521 |
| 126 | Met | (126) 18 | (2) 573 |
| 127 | Gly | (127) 19 | (3) 102 |
| 128 | Pro | (128) - | (4) 467 |
| 129 | Ser | (129) 12 | (5) 504 |
| 130 | Ala | (130) - | (6) 404 |
| 131 | Gln | (131) - | (7) 401 |
| 132 | Ala | (132) - | (8) 266 |
| 133 | Ile | (133) - | (9) 125 |
| 134 | Thr | (134) - | (10) 28 |
| 135 | Ser | (135) - | (11) 168 |
| 136 | Leu | (136) - | (12) 198 |
| 137 | Pro | (137) - | (13) 215 |
| 138 | Tyr | (138) - | (14) 71 |
| 139 | Arg | (139) - | (15) 189 |
| 140 | Gly | (140) - | (16) 198 |
| 141 | Gly | (141) - | (17) 47 |
| 142 | Thr | (142) - | (18) 114 |
| 143 | Asn | (143) - | (19) 136 |
| 144 | Leu | (144) - | (20) 192 |
| 145 | Gly | (145) 580 | (21) 26 |
| 146 | His | (146) 298 | (22) 98 |
| 147 | Asp | (147) 559 | (23) 16 |
| 148 | Lys | (148) 677 | |
| 149 | Ile | (149) 501 | |
| 150 | Thr | (150) 26 | LE-20 (1) 122 |
| 151 | Lys | (151) 474 | (2) 97 |
| 152 | Leu | (152) 162 | (3) 156 |
| 153 | Ile | (153) - | (4) 119 |
| 154 | Asn | (154) - | (5) 110 |
| 155 | Phe | (155) - | (6) 90 |
| 156 | Asn | (156) - | (7) 108 |
| 157 | Phe | (157) - | (8) 135 |
| 158 | Leu | (158) - | (9) 40 |
| 159 | Ser | (159) - | (10) 58 |
| 160 | Cys | (160) - | (11) 51 |
| 161 | Leu | (161) - | (12) 44 |
| 162 | Arg | (162) - | (13) 52 |
| 163 | Arg | (163) - | (14) 105 |
| 164 | Arg | (164) - | (15) 22 |
| 165 | Asp | (165) - | (16) 30 |
| 166 | Ser | (166) - | (17) 11 |
| 167 | His | (167) - | (3) 160 |
| 168 | Lys | (168) - | (4) 241 |
| 169 | Ile | (169) 263 | (5) 60 |
| 170 | Asp | (170) 275 | |
| 171 | Ser | (171) 45 | |
| 172 | Phe | (172) 128 | |
| 173 | Leu | (173) 100 | |
| 174 | Lys | (174) 37 | |
| 175 | Val | (175) - | |
| 176 | Leu | (176) - | |
| 177 | Arg | (177) - | |
| 178 | Cys | (178) - | |
| 179 | Arg | (179) - | |
| 180 | Ala | (180) - | |
| 181 | Ala | (181) - | |
| 182 | Lys | (182) - | |
| 183 | Met | (183) 1002 | |
| 184 | Gln | (184) 843 | |
| 185 | Pro | (185) 451 | |
| 186 | Glu | (186) 625 | |
| 187 | Met | (187) 319 | |
| 188 | Cys | (188) 166 | |

Table 5. Carboxypeptidase A digestion of carboxamidomethylated tPR1188

| Time (min) | Residues/mole | |
|------------|---------------|---------------------------------|
| | Met(187) | Carboxamidomethyl-cysteine(188) |
| 10 | - | 0.09 |
| 30 | 0.07 | 0.24 |
| 120 | 0.15 | 0.71 |

CT-10

CT-9

CT-21