hormonal proteins and peptides

PROLACTIN

EDITED BY CHOH HAO LI

volume 8

HORMONAL PROTEINS AND PEPTIDES

Edited by CHOH HAO LI

The Hormone Research Laboratory
University of California
San Francisco, California

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Preface

Prolactin is remarkable in its broad spectrum of biological activities among the vertebrates. It acts as a synergist with steroid hormones in the sequential development of ductal and lobuloalveolar components of the mammary tree (mammogenic action). Secretory activity is also dependent upon the action of prolactin in synergism with adrenal hormones to initiate milk production (lactogenic action) and to maintain and augment it (galactopoietic action).

In addition to its striking actions in stimulating crop "milk" formation in birds, prolactin induces incubation behavior in the ring dove. Prolactin causes increased body weight in tadpoles. For freshwater fish, prolactin is essential for the survival of osmotic stress. In efts, prolactin induces the land-living stage of this species to return prematurely to water. The water-drive activity of prolactin is also seen in other salamanders.

Extensive work in rats has clearly established that prolactin plays an important role in the development and progression of certain mammary tumors. Many breast tumors in experimental animals can be shown to be prolactin dependent, accelerating in rate of growth when prolactin levels are raised and regressing after hypophysectomy. Evidence has also been obtained showing that prolactin promotes the spontaneous development of breast tumors in mice.

This volume opens with a chapter on the chemistry of prolactin. The role of prolactin on normal mammary gland growth and function is reviewed authoritively in the second chapter by Elias who described in 1957 for the first time the organ culture technique to study the action of prolactin *in vitro*. In Chapter 3, Clifton and Furth consider prolactin effects in tumor induction and growth. Furth was one of the leaders in the field of cancer research for many years.

The fourth chapter reviews the present status of the comparative endocrinology and evolutionary biology of prolactin. This chapter, by Clarke and Bern, is probably the most comprehensive and critical treat-

ment of the subject in recent years. Bern is one of the leading figures in comparative endocrinology responsible for the development of our knowledge on comparative biology of prolactin.

In the concluding chapter, Greep comments on the work of two pioneers in reproductive biology. An outstanding investigator in reproductive endocrinology, Greep knew Hisaw and van Dyke very well as a co-worker in their laboratories for a number of years. The important advances made for the last decade in gonadotropins including prolactin, relaxin, lutropin, and follitropin, are indebted to the pioneering work of Hisaw and van Dyke as well as Greep.

Once more, I wish to thank the staff of Academic Press for their assistance and cooperation in the preparation of this volume.

Choh Hao Li

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The Chemistry of Prolactin

CHOH HAO LI (1), Hormone Research Laboratory, University of California, San Francisco, California 94143

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I. Introduction*

The first indication that a hormone of the pituitary gland might effect lactation in mammals came from the experiments of Stricker and Grueter in 1928. At the suggestion of their mentor, P. Bouin, Stricker and Grueter (1928) used rabbits that had been exposed to the secretions of the corpora lutea of pseudopregnancy and showed that bovine pituitary extracts induced milk secretion in these oophorectomized animals. About the same time, Corner (1930), working independently on the same problem, discovered that milk secretion could be induced in the mammary glands of adult castrated virgin female rabbits by injection of sheep pituitary extracts. In 1932, Riddle *et al.* (1932) discovered a fraction from bovine pituitary extracts capable of stimulating the crop sac growth in pigeons, and named it prolactin. Subsequent work indicated that this effect in birds is due to the same hormone that induces lactation in rats and rabbits.

Lactogenic activity has since been reported to be present in the pituitary extracts of various species, including humans, sheep, cattle, pigs, horses, whales, rabbits, cats, rats, guinea pigs, mice, fish, amphibians, reptiles, and birds. During the period of 1937–1955, highly purified prolactin preparations were obtained from cattle and sheep pituitary glands by a number of investigators (Li, 1957*a*). In recent years, it has also been obtained in highly purified form from pig (Eppstein, 1964), rat (Ellis *et al.*, 1969), dog (Papkoff, 1976), chicken (Scanes *et al.*, 1975), fish (Farmer *et al.*, 1977), and man (Lewis *et al.*, 1971).

The primary structures are known ovine (Li *et al.*, 1970), bovine (Wallis, 1974), porcine (Li, 1976), and human (Shome and Parlow, 1977) prolactins. However, for the last 40 years, extensive structure–function and physiological studies of prolactin have been used chiefly on the ovine hormone. This chapter presents mainly the current knowledge on the chemistry of ovine prolactin.

II. Isolation Procedures

A. Ovine Prolactin

We have been employing the following procedure routinely for many years for the isolation of ovine prolactin in highly purified form for various chemical and biological investigations. One kilogram of sheep pituitaries

*Abbreviations: hPRL, human prolactin; oPRL, ovine prolactin; pPRL, porcine prolactin; GH, growth hormone; HGH, human growth hormone, SGH, sheep GH; CD, circular dichroism; HCS, human choriosomatomammotropism.

is ground with a minimal amount of water to a frozen slurry. Three volumes of prechilled (−20° C) acetone, containing 25 ml of concentrated HCl per liter, is added and the suspension is stirred for 1 hour in the cold and then filtered. Five volumes of prechilled acetone is added to the filtrate, resulting in the formation of a precipitate. After the precipitate has settled, most of the acetone can be decanted. The precipitate is collected by filtration, air dried, and ground to a powder. A yield of 25–30 gm is obtained per kilogram of glands. The material is dissolved in 1 liter of water and saturated sodium chloride solution is added to a concentration of 6 percent saturation. The pH is adjusted to 3.0 and the formed precipitate (crude lactogenic hormone fraction) is removed by centrifugation. This NaCl precipitate (20 gm) is dissolved in water adjusted to pH 6.3 with 1.0 *N* NaOH. After standing in the cold for 1–2 hours the precipitate that forms is removed by centrifugation. The supernatant fluid is adjusted to pH 5.6 with 1.0 *N* HCl. The resultant precipitation is dissolved in water and isoelectric precipitation is repeated twice. Final purification of this fraction is accomplished by exclusion chromatography on a Sephadex G-100 column (215 × 145 cm) in 0.1 *M* $NaHCO_3$. The contents of the major peak are combined, dialyzed, and lyophilized. The lyophilized product (0.5 gm) is the prolactin monomer (Squire *et al.*, 1963). The product is found to have a potency* of 42 IU/mg with 95% confidence limits of 22–90 IU/mg when compared with NIH-PS10 (26 IU/mg) as assayed by the pigeon crop sac test (Nicoll, 1962).

B. Human Prolactin

The isolation of human prolactin has been reported by Lewis *et al.* (1971) from fresh-frozen glands and by Hwang *et al.* (1972) from acetone-dried glands. Recently, Rathman and Saxena (1977) developed a procedure to obtain hPRL of high potency in good yields from acetone-preserved glands which had been used for the purification of other pituitary hormones. This procedure is briefly outlined as follows: The residue obtained after extraction of the glycoprotein hormones from pituitary acetone powder (Hartree, 1966) is extracted for HGH by the Raben method (Raben. 1957). After precipitation of HGH at 50% ethanol, the supernatant was adjusted to 85% (v/v) ethanol. The precipitate obtained contained a significant quantity of HGH and a little hPRL. The supernatant was made 50% in acetone to precipitate the "PRL fraction." This fraction was further purified by gel filtration on Sephadex G-100 in 0.1 *M* NH_4HCO_3 of pH 8.5, and followed by ion exchange

*Personal communication of C. S. Nicoll, Feb. 2, 1978.

chromatography on DEAE-cellulose in 0.01 *M* Tris-HCl buffer of pH 8.6 with stepwise eluation of increasing NaCl concentrations. The PRL fraction from DEAE-cellulose column was next submitted to isoelectric focusing to yield 23 mg prolactin from 1000 gm (approximately 10,000) pituitary acetone powder with a potency of 37 IU/mg.

It is of interest to compare this yield with that of other investigators. Hwang *et al.* (1972) obtained 16 mg PRL containing 30 IU/mg from 10,000 acetone-dried glands whereas Lewis *et al.* (1971) obtained 85 mg of the hormone with a potency of 22 IU/mg from 10,000 fresh-frozen glands. Human PRL was less electronegative than HGH at pH 9.5 and had a molecular weight of about 22,000 (Lewis *et al.*, 1971).

The procedure of Lewis *et al.* (1971) for the isolation of hPRL is briefly described here. Sixty gm of fresh-frozen human pituitary glands (about 120 glands) were homogenized with 300 ml of saline and centrifuged at 20,000 *g* for ½ hour; the sedimented tissue was resuspended in 200 ml saline and centrifuged again. The insoluble material was stirred overnight with 300 ml of 0.5 *M* $NaHCO_3$–$NaCO_3$ buffer of pH 10 containing 2×10^{-3} *M* *p*-aminobenzamidine; the mixture was then frozen, thawed, and centrifuged at 20,000 *g* for ½ hour. The sediment from this was resuspended in 200 ml of buffer and again centrifuged after freezing and thawing. These two supernates were combined, the pH lowered to 8.5 with HCl, and the cloudy solution concentrated on an ultrafiltration membrane (Diablo UM-10, Aminco) to a volume of 80 ml. Twenty-milliliter aliquots of the concentrated extract were chromatographed on a column (5 × 90 cm) of Sephadex G-150 with 0.01 *M* NH_4HCO_4. The fraction containing HGH and hPRL was combined, concentrated on a UM-10 membrane to 15 ml, and chromatographed on the same column. The HGH-hPRL fraction was concentrated on a UM-10 membrane to 15 ml and used for DEAE-cellulose chromatography in 0.01 *M* NH_4HCO_3 and 0.2 *M* NH_4HCO_3. The hPRL fraction was again concentrated and rechromatographed on DEAE-cellulose. The hPRL fraction was lyophilized and yielded 1.1 mg. The purified hormone had a potency of 22 IU/mg in the pigeon crop sac assay (Nicoll, 1962) when compared with NIH-PS8 (28 IU/mg). The somatotropin activity of hPRL was estimated by the tibia assay (Greenspan *et al.*, 1949) to be about 0.4 USP U/mg. The molecular weight of hPRL was estimated to 22,000 (Lewis *et al.*, 1971).

III. Characterization of Ovine and Porcine Prolactin

The isolation of porcine prolactin (Li, 1976) from acid–acetone extracts of porcine pituitary glands was carried out by the procedure for the ovine

hormone described above. Approximately 0.8 gm of pPRL monomer was obtained from 1 kg of fresh glands. Bioassay of the final product showed its potency to be 31.5 IU/mg in the pigeon crop sac test.

The porcine hormone behaves as a homogeneous protein with a Stokes radii of 24.9 Å in a calibrated Sephadex column at pH 8.2. Sedimentation velocity experiments at pH 8.2 again indicate pPRL is a homogeneous protein with no concentration dependence in sedimentation coefficient between 2 and 8 mg/ml protein (Bewley and Li, 1975). The $s_{20°,w}$ value of 2.18 S was found to be the same as previously reported for monomer ovine protein (Squire *et al.*, 1963). When this value is combined with the Stokes radius and specific volume, the computed value for the molecular weight of pPRL (23,000) is in excellent agreement with that obtained by ultracentrifuge data, indicating good consistency between the sedimentation and exclusion chromatographic behavior of the hormone. High-speed sedimentation equilibrium at pH 10.35 indicated homogeneity of the preparation with an average molecular weight of 22,400.

Free-boundary electrophoresis indicated pPRL is also electrophoretically homogeneous. The fact that the isoelectric point (p*I*, 5.85) of pPRL is slightly more basic than that of oPRL (p*I*, 5.73) is in accord with the amino acid composition of the two hormones (Li *et al.*, 1970; Li, 1976). Although both contain the same total number of carboxyl groups, pPRL has two more arginine and one more histidine residue than the ovine hormone.

The solubility behavior (Bewley and Li, 1975) of both pPRL and oPRL in ammonium sulfate solutions indicates that oPRL is more soluble than the porcine hormone.

A. Circular Dichroism

The CD spectrum of ovine prolactin in pH 8.2 Tris buffer (Bewley and Li, 1972) shows a strong negative band at 223 nm and a second slightly weaker band around 209 nm. It is estimated that the α-helix content is 55%, in agreement with the value reported by Aloj and Edelhoch (1970). The spectrum in the region of side-chain absorption shows that it contains a single asymmetric positive band with an apparent maximum around 297–298 nm and badly resolved system of negative bands showing at three negative maxima at 281–282, 275–276, and 268 nm.

The effect of three solvents on the amide bond CD spectra (Bewley and Li, 1972) of ovine prolactin has also been investigated. Five molar guanidine hydrochloride results in a marked loss of negative dichroism over the narrow spectral range in which accurate measurements can be made. Over the same limited spectral range, 50% acetic acid produces a

much smaller loss and is only slightly more effective in this respect than glycine buffer of pH 3.6. The negative peak occurring at 223 nm in the native protein has been shifted to 221 nm along with a 20–25% decrease in intensity. Repeated measurements have shown this small blue shift to be quite reproducible. A similar shift is not observed for the peak at 209 nm although its intensity is decreased by about 15%, resulting in a reversal in the relative intensity of the two peaks. If these solvents are removed by dialysis against 0.1 *M* Tris buffer of pH 8.2, the CD spectra of all three samples return to essentially that of the native protein.

The effect of these solvents on the side-chain dichroism gives only weakly negative band between 295 and 260 nm in 5 *M* guanidine hydrochloride. Both the glycine buffer of pH 3.6 and 50% acetic acid result in the complete loss of the positive band at 298 nm and increased resolution of the two negative bands around 268–269 nm and 261–262 nm. The 50% acetic acid has no significant effect on the spectrum between 284 and 268 nm, but the glycine buffer produces a small increase in the negative bands in this region. Following dialysis, all three samples show side-chain CD spectra which are at most only very slightly altered from those of the native protein.

B. Spectrophotometric Titration

The spectrophotometric titration of porcine prolactin in 0.1 *M* KCl was performed by the difference spectra technique (Bewley *et al.*, 1969). The family of curves generated during the titration shows absorption maxima at 295 nm. Plotting the change in absorption at 295 nm vs pH produces the ionization curve which shows that only six of the seven tyrosine residues in porcine prolactin can be titrated at pH 13.1. The pK_a of these six groups, estimated from the midpoint of the ionization curve, was found to be 11.15. These data are quite comparable to those reported for ovine prolactin in which only six of the seven tyrosyls could be titrated in 0.15 *M* KCl, with a pK_a, of 11.2–11.3 (Ma *et al.*, 1970).

C. Fluorescence Emission Spectra

The fluorescence emission spectra of both ovine and porcine prolactin have been measured (Bewley and Li, 1975) at 27° C in Tris–HCl buffer (pH 8.2), as well as those of porcine prolactin in acetate buffer (pH 4.0). Excitation was effected at 292 nm in order to limit the emission predominantly to tryptophan. In addition, this wavelength was found to be the excitation maximum of both proteins in the Tris–HCl buffer. The wavelengths of maximum emission were found to be 337.9 ± 1.0 nm for por-