Structure of Chicken Hypothalamic Luteinizing Hormone-releasing Hormone

II. ISOLATION AND CHARACTERIZATION*

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Avian luteinizing hormone-releasing hormone (LH-RH) has been isolated from 249,000 chicken hypothalami and shown to differ structurally from mammalian hypothalamic LH-RH. Purification was achieved by acetic acid extraction, anti-LH-RH affinity chromatography, and cation exchange and reverse phase high performance liquid chromatography. The isolated peptide eluted as a single peak on reverse phase high performance liquid chromatography. Acid hydrolysis of the peptide yielded integral molar ratios of amino acids and a composition identical with that of mammalian decapeptide LH-RH, except for the presence of an additional glutamic acid residue and the absence of arginine. The isoelectric point of chicken LH-RH (7.3) is consistent with the glutamic acid representing a glutamine residue. We therefore synthesized [Gln]LH-RH and established that it has chromatographic properties identical with natural chicken LH-RH. These studies indicate that the structure of chicken hypothalamic LH-RH is: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH₂.

The decapeptide LH-RH* (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂), originally isolated from porcine hypothalami (Matsuo et al., 1971a) and later synthesized (Matsuo et al., 1971b), appears to have lower gonadotropin-releasing activity in submammalian vertebrates than in mammals. Crude hypothalamic extracts from birds, reptiles, amphibians, and fish, however, contain significant gonadotropin-releasing activity, and LH-RH-like immunoactivity has been demonstrated in the hypothalami of these vertebrates by radioimmunoassay and immunocytochemistry (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981). We have shown that immunoreactive amphibian hypothalamic LH-RH is identical with the mammalian decapeptide LH-RH, except for the presence of an additional glutamic acid residue and the absence of arginine. The isoelectric point of chicken LH-RH (7.3) is consistent with the glutamic acid representing a glutamine residue. We therefore synthesized [Gln]LH-RH and established that it has chromatographic properties identical with natural chicken LH-RH. These studies indicate that the structure of chicken hypothalamic LH-RH is: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH₂.

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1 The abbreviations used are: LH-RH, luteinizing hormone-releasing hormone; HPLC, high performance liquid chromatography.
Isolation of Chicken Hypothalamic LH-RH

TABLE I
Summary of purification scheme for chicken hypothalamic LH-RH

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Dry weight</th>
<th>Total peptide</th>
<th>Immunoreactive LH-RH</th>
<th>Recovery of LH-RH at each step</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyophilized hypothalami</td>
<td>163.3</td>
<td>57,120.0</td>
<td>g</td>
<td>%</td>
</tr>
<tr>
<td>2 N acetic acid extract</td>
<td>22.2</td>
<td>7,461.0</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>0.100</td>
<td>36.2</td>
<td>µg</td>
<td></td>
</tr>
<tr>
<td>Cation exchange HPLC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse phase HPLC</td>
<td>0.016</td>
<td>17.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Determined by amino acid analysis.

FIG. 1. Affinity chromatography of immunoreactive chicken LH-RH on a Sepharose-anti-LH-RH 1076 column. Aliquots of fractions were assayed for LH-RH immunoreactivity with antiserum R-42, and for Folin-Lowry peptide using bovine serum albumin as standard. NH4Ac, ammonium acetate; HAc, acetic acid. One of 10 affinity runs is shown here; a similar pattern was seen in all column runs.

Fractions of 1.5 ml were collected and aliquots of 5 µl were taken for radioimmunoassay. Immunoreactive fractions from all four runs were pooled and lyophilized.

Radioimmunoassay—LH-RH was measured as described in the preceding paper (King and Millar, 1982b). Antiserum R-42 was used throughout the study since it yields chicken hypothalamic LH-RH displacement curves parallel to that of mammalian LH-RH and apparently estimates chicken LH-RH nearly quantitatively (King and Millar, 1982b). For effective binding, the antiserum requires both NH2 and COOH termini and tolerates certain alterations in the middle of the decapeptide (Nett et al., 1973; Copeland et al., 1979).

Lyophilized aliquots of extracts and chromatographic column fractions were reconstituted in phosphate-buffered saline containing gelatin (0.04 M phosphate, 0.15 M NaCl, 0.01 M disodium ethylenediaminetetraacetic acid, 0.015 M NaN3, pH 7.0, with 0.1% gelatin) and assayed for LH-RH immunoreactivity.

Amino Acid Analysis—Purified immunoreactive LH-RH (0.5 µg) was hydrolyzed in 100 µl of constant boiling 6 N HCl containing 2% thioglycolic acid in sealed evacuated tubes at 110 °C for 16 h. The hydrolysate was lyophilized, reconstituted in 0.16 M lithium citrate buffer, pH 2.2, and subjected to amino acid analysis (Beckman Instruments Model 121-MB).

Comparison of Natural Chicken LH-RH with Synthetic [Gln8] LH-RH—[Gln8]LH-RH was synthesized by the solid phase method of Merrifield (1963) and its structure was verified by chemical analyses. The chromatographic properties of synthetic [Gln8]LH-RH on cation exchange and reverse phase HPLC were determined using the methods described above for the purification of natural chicken LH-RH. The isoelectric points of natural chicken LH-RH and synthetic [Gln8]LH-RH were determined on polyacrylamide gel rods as described in the preceding paper (King and Millar, 1982b).

RESULTS

Immunoreactive chicken hypothalamic LH-RH was purified 2 million-fold using the scheme outlined in Table I. The hypothalamic acetic acid extract contained 7.5 g of total peptide and 33.7 µg of immunoreactive LH-RH. In affinity chromatography, nonimmunoreactive peptides were eluted with 0.5 M ammonium acetate and immunoreactive LH-RH was then eluted with 1.5 N acetic acid (Fig. 1). The affinity-purified immunoreactive LH-RH was subjected to cation exchange HPLC which revealed a major peak of immunoreactive LH-RH at 7-8 min (11.2-12.8 ml) and an earlier eluting minor immunoreactive peak at 4 min (6.4 ml) (Fig. 2). The major immunoreactive LH-RH component was further purified by reverse phase HPLC. A single peak of immunoreactive LH-RH co-eluted with a single sharp absorbance peak at 23-24 min (34.5-36.0 ml) (Fig. 3), indicating that the isolated peptide was essentially pure.

The LH-RH was homogeneous, as judged by amino acid analysis (Table II), demonstrating integral molar ratios of...
The isolation of LH-RH from hypothalami presents difficulties.

Amino acid analysis of the acid-hydrolyzed peptide demonstrated the presence of the same ratio of amino acids present in mammalian LH-RH except for an absence of arginine and an additional glutamic acid residue. The isoelectric point of chicken LH-RH (7.3) is consistent with a neutral amino acid replacing arginine at position 8 in the peptide. Thus, glutamic acid is most likely represented as glutamine in the peptide. In view of the structure of LH-RH (blocked NH$_2$ and COOH termini and the presence of a proline residue at position 9), attempts to demonstrate glutamine by amino acid analysis of enzymic hydrolysates of the peptide and by microsequencing techniques were unsuccessful and were not pursued further owing to scarcity of material. For solid phase microsequencing, the COOH-terminal Gly-10-NH$_2$ and NH$_2$-terminal pGlu-1 were first removed by incubation with post-proline-cleaving enzyme and pyroglutamimidopeptidase. Various approaches to coupling the COOH-terminal Pro-9 to an aminopropyl glass were attempted but all were unsuccessful. We therefore adopted the conventional liquid phase sequencing technique after mild chymotrypsin cleavage of 2 µg of purified chicken LH-RH to generate presumed fragments LH-RH (1-5) and LH-RH (6-10). On Edman degradation, residues 6 and 7, glycine and leucine, were identified in the first and second cycles, respectively. The crucial position 8 amino acid residue was undetectable in the third cycle.

Indirect studies strongly support the conclusion that the only difference between chicken and mammalian LH-RH resides in the replacement of glutamine for arginine in the 8 position with the retention of all other structural features including pGlu-1 and Gly-10-NH$_2$. The interaction of region-specific antisera with the peptide, and studies on the effects of chemical modification of specific amino acid residues and cleavage of specific peptide bonds on immunoreactivity, all support this conclusion (King and Millar, 1982b). It is also pertinent that a common neutral amino acid substitution for arginine is glutamine (Dayhoff et al., 1972). Glutamine is also an appropriate neutral amino acid substitution for arginine if some semblance of the combined unit of hydrogen bond interactions between the side chains of His-2, Tyr-5, and Arg-8 in mammalian LH-RH is to be retained (Shinitzky and Fridkin, 1976). These authors believe this combined unit is important for biological activity. Finally, we have synthesized the putative sequence of chicken LH-RH by conventional solid phase methodology and shown that it has identical physicochemical properties to the natural chicken peptide. In contrast, other position 8-substituted LH-RH analogs had distinctly different properties.

The nature of hypothalamic LH-RH in submammalian vertebrates has been studied by radioimmunoassay and chromatography (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981). Several reports indicated a structural identity between mammalian hypothalamic LH-RH and chicken and frog hypothalamic LH-RHs (Deery, 1974; Jeffcoate et al., 1974; Alpert et al., 1976) while others reported differences in the physicochemical and biological properties of chicken and teleost fish hypothalamic LH-RHs (Jackson, 1971a, 1971b; Hattori et al., 1980; Jackson, 1981; Barnett et al., 1982). On the basis of chromatographic properties and using region-specific antisera, we demonstrated that avian, reptilian, and piscine hypothalamic LH-RHs differed structurally from...
mammalian hypothalamic LH-RH, the difference being in the region Gly-6-Leu-7-Arg-8 (King and Millar, 1979, 1980, 1981). Amphibian hypothalamic LH-RH was, however, identical with the mammalian hypothalamic peptide (King and Millar, 1979, 1980, 1981) and this has been confirmed by recent studies on the amino acid composition of frog brain LH-RH (Rivier et al., 1981). More recently, we have shown in indirect studies on partially purified material that chicken hypothalamic LH-RH differs structurally from the mammalian peptide by the substitution of glutamine for arginine in position 8 (King and Millar, 1982b), which accords with the present study.

Since hypothalamic LH-RHs from birds, reptiles, and teleost fish behave identically on CM32 carboxymethylcellulose cation exchange chromatography and reverse phase HPLC as well as in their interaction with region-specific antisera (King and Millar, 1979, 1980, 1981), it is possible that a substitution of glutamine for arginine of mammalian LH-RH is the characteristic structural feature of the peptide in all of these vertebrate classes. However, the HPLC system used in our early studies may not have been adequate to resolve certain neutral amino acid substitutions in position 8 from each other, and other position 8-substituted analogues may be present in reptiles and fish. Thus, [Gln8]LH-RH or another analogue with a conservative neutral amino acid substitution in position 8 is probably the ancestral molecule, and a mutation to [Arg8] LH-RH occurred in a line common to amphibians and mammals in accordance with a contemporary phylogenetic scheme (Licht et al., 1977; King and Millar, 1979).

Synthetic mammalian LH-RH has been found to exhibit gonadotropin-releasing activity in amphibians but has poor or no such activity in birds, reptiles, and fish (for reviews, see Ball, 1981; Jackson, 1981; King and Millar, 1981). It is likely that this phenomenon reflects structural differences in pituitary gonadotroph receptors related to the structural difference of LH-RH in these vertebrate classes.

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