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 decapptide LH-RH, except for the presence of an additional glutamic acid residue and the absence of arginine. The isolectric 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, amphabians, and fish, however, contain significant gonadotropin-releasing activity, and LH-RH-like immunoreactivity 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 glutamine (King and Millar, 1982a, 1982b). This conclusion describes the isolation and amino acid composition of homogeneously pure chicken hypothalamic LH-RH and confirms that glutamine substitutes for the arginyl residue at position 8 in mammalian hypothalamic LH-RH.

MATERIALS AND METHODS

Extraction—Fragments of 249,000 chicken hypothalami consisting of the median eminence and pituitary stalk were dissected, frozen on dry ice, and lyophilized. Three batches of lyophilized hypothalami were defatted with petroleum ether (40-60 °C) at 4 °C and extracted with 2 N acetic acid at 4 °C using an Ultraturrax homogenizer and then a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 18,000 × g for 1 h at 4 °C, and the insoluble residue was re-extracted four times. The supernatants from the five extractions were pooled, and aliquots were taken for radioimmunoassay and for total peptide determination using bovine serum albumin as standard (Lowry et al., 1951). The supernatant extract was then lyophilized.

Affinity Chromatography—Antiserum 1076, which requires residues within the region Trp-3 to Pro-9 of synthetic mammalian LH-RH for effective binding (see King and Millar, 1982b), was used. The γ-globulin fraction of the antiserum obtained after ammonium sulfate precipitation was immobilized on cyanogen bromide-activated Sepharose 4B (Pharmacia) at a concentration of 0.3 ml of antiserum equivalents of γ-globulin/g of Sepharose. The retention capacity of the Sepharose-anti-LH-RH 1076 column (3.5 × 26 cm) was 8.3 μg of synthetic mammalian LH-RH. Due to this limited retention capacity, it was necessary to process the supernatant extract in 10 separate runs. The lyophilized supernatant extract was reconstituted in 0.5 ml ammonium acetate, pH 7.0, and cycled through the column for 8 h. The column was washed with 0.5 ml ammonium acetate to remove unbound peptides, and the bound material was eluted with 1.5 ml acetic acid. Aliquots of fractions were taken for radioimmunoassay and for Folin-Lowry total peptide determination. Fractions with LH-RH immunoactivity from all 10 runs were pooled and lyophilized.

High Performance Liquid Chromatography—Two different systems were used on a Model SP 3500B liquid chromatograph (Spectra-Physics) equipped with a Model 770 spectrophotometric detector (Spectra-Physics). The first HPLC system consisted of a Partisil 10/25 SCX cation exchange column (0.4 x 25 cm; Whatman) and a mobile phase of 10% ethanol in 0.05 M ammonium acetate, pH 5.6. Lyophilized affinity-purified immunoreactive LH-RH was reconstituted in HPLC buffer and eluted in 12 separate runs. Flow rate was 1.6 ml/min. Absorbance at 280 nm was monitored at 0.2 unit full scale. Fractions of 1.6 ml were collected and aliquots of 5 μl were taken for radioimmunoassay. Immunoreactive fractions from all 12 runs were pooled and lyophilized. The second HPLC system consisted of a Spherisorb ODS reverse phase column (0.4 × 25 cm; 5-μm particle size; Phase Separations) and a mobile phase of 2% acetonitrile in 0.01 M ammonium acetate, pH 4.0. Lyophilized immunoreactive LH-RH from the cation exchange HPLC system was reconstituted in HPLC buffer and eluted in four separate runs. Flow rate was 1.5 ml/min. Absorbance at 280 nm was monitored at 0.04 unit full scale.
Isolation of Chicken Hypothalamic LH-RH

TABLE I

<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></td>
<td></td>
</tr>
<tr>
<td>2 N acetic acid extract</td>
<td>22.2</td>
<td>7,461.0</td>
<td>33.7</td>
<td>100</td>
</tr>
<tr>
<td>Affinity chromatography</td>
<td>0.100</td>
<td>36.2</td>
<td>24.4</td>
<td>73</td>
</tr>
<tr>
<td>Cation exchange HPLC</td>
<td>19.5</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reverse phase HPLC</td>
<td>0.016&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.4</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by amino acid analysis.

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 in view of the low total content and concentration of the peptide. This is particularly so in the avian hypothalamus in view of the low total content and concentration of the peptide and its physicochemical properties to the natural chicken peptide. In contrast, other position 8-substituted LH-RH analogues 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; Jeffcoat 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 LH-RH except for an absence of arginine and with 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 hydrolyses 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 pyroglutamyl aminopeptidase. Various approaches to coupling the COOH-terminal Pro-9 to aminopropyl glass were attempted but all were unsuccessful. We therefore adopted the conventional liquid phase sequenencing 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 immunoactivity, 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 analogues had distinctly different properties.

**DISCUSSION**

This report describes the first biochemical characterization of hypothalamic LH-RH from submammalian vertebrates. The isolation of LH-RH from hypothalami presents difficulties in view of the low total content and concentration of the peptide. This is particularly so in the avian hypothalamus in which the immunoreactive LH-RH content is considerably lower than in mammals. In the hypothalami of the adult chicken and pigeon, the content of immunoreactive LH-RH as measured with antiserum 422, which requires the NH$_2$ and COOH termini of the molecule for binding and which cross-reacts most with avian LH-RH, was 2.8-4.3 ng (King and Millar, 1980). In the present study using hypothalami from immature chickens and dissection of a more discrete region (median eminence and pituitary stalk) to simplify purification, a number of chromatographic systems were tested in studies preliminary to the final purification scheme. From these we selected a combination of anti-LH-RH affinity chromatography followed by only two HPLC systems which were sufficient to yield homogeneously pure peptide totalling 17.4 µg. This represented a purification of 2 million-fold with an overall recovery from starting material of 51%. Contamination with non-LH-RH material as determined by amino acid analysis was less than 1%.

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 with 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 hydrolyses 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 pyroglutamyl aminopeptidase. Various approaches to coupling the COOH-terminal Pro-9 to 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 immunoactivity, 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 analogues had distinctly different properties.
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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Structure of chicken hypothalamic luteinizing hormone-releasing hormone. II. Isolation and characterization.

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