Follitropin (FSH) receptors were solubilized from pure light membranes of bovine calf testis, using an optimum detergent to protein ratio of 0.01. The soluble FSH receptor fraction was gel filtered through Sepharose 6B to isolate an active fraction (6B-Fr-1) which behaved as a complex of FSH receptor and Gs protein. The 6B-Fr-1 was concentrated by ultrafiltration and further purified by sequential Sepharose 4B gel filtration, DEAE-cellulose chromatography (to separate the receptor from Gs protein), and wheat germ lectin affinity chromatography. The purified receptor had an FSH-binding capacity of \( \sim 3.47 \text{ nmol/mg of protein} \) with a \( K_d \) of \( 1.9 \times 10^{-10} \) M. Yield was 526 \( \mu \text{g/1.5 kg} \) tested. Radioiodinated, as well as unlabelled purified FSH receptor, migrated on sodium dodecyl sulfate-polyacrylamide gels as a single major band of \( M_r \sim 240,000 \). This band was not affected by \( 8 \% \) urea treatment prior to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, but treatment with dithiothreitol induced the loss of the 240-kDa band, with appearance of an \( M_r \sim 60,000 \) band. The availability of highly purified, stable FSH receptor should allow direct studies on its structure-function relationships.

**DISCUSSION**

There are only a few studies on the purification of follitropin receptor (11, 12). Although in earlier studies a significant amount of soluble receptor was recovered by using hormone-affinity chromatography, the isolated receptor from bovine testis was either inactive (12) or low in activity (11). The presence of low concentrations of follitropin receptors in crude membranes as well as poor stability and yield after solubilization by detergents, have been the main problems experienced in those studies. In this report, we described a new procedure that overcame these problems and allowed the preparation of substantial quantities of follitropin receptor in a highly purified and stable state.

Testis plasma membranes contain both high affinity and low capacity, as well as low affinity and high capacity FSH-binding sites (13, 30). The occupancy of high affinity FSH-binding sites leads to the activation of adenylate cyclase (1, 4). Bovine calf testis homogenate was, therefore, centrifuged to remove low affinity and high capacity FSH-binding sites into a 7,000 \( \times \) g sediment fraction containing heavier plasma membranes. The 7,000 \( \times \) g supernatant containing only high affinity labeling techniques have yielded variable results (7–10). A widely used nonionic detergent, Triton X-100, was utilized in earlier studies (11, 12) for the solubilization and purification of follitropin receptor from calf testes. The low concentration of follitropin receptors in crude testis membranes and the lability of the receptor once solubilized by detergents have greatly hampered efforts at its purification in quantities sufficient for chemical characterization and structure-function studies.

Recently, we have used a new approach for the solubilization of follitropin receptors from bovine calf testis membranes. The approach is dependent on isolation of receptor-enriched light membrane fractions from bovine calf testis homogenates and optimizing conditions for solubilization of follitropin receptors by utilizing low ratios of Triton X-100 to membrane protein, with removal of interfering lipids by petroleum ether extraction (13). The resulting detergent-soluble fraction contained stable and functional follitropin receptors (3, 13) and represented an ideal precursor for its further purification. We report here the purification of the follitropin receptor from bovine testis.

**MATERIALS AND METHODS AND RESULTS**

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1 Portions of this paper (including "Materials and Methods," "Results," and Figs. 1–13) 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.

2 The abbreviations used are: FSH, follitropin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Gpp(NH)p, guanosine 5'-imidotriphosphate; bLH, bovine lutropin; hCG, human chorionic gonadotropin; TSH, thyrotropin.
affinity and low capacity FSH-binding sites was further processed.

An important feature of follitropin receptor activity in the crude testicular tissue homogenate is its rapid decline due to proteolytic degradation and the presence of follitropin receptor-binding inhibitors (28, 29). These inhibitors, if not removed, co-solubilize and co-purify with the receptor and are responsible for decreasing the follitropin-binding activity of the detergent-solubilized receptor. An initial concentration of the spermatozoa supernatant by Amicon DC-10 ultrafiltration was carried out in the presence of a protease inhibitor, sodium p-hydroxymercuribenzoate. This rendered FSH receptor activity in the resulting concentrate more stable and also removed significant amounts of inactive proteins as well as FSH receptor-binding inhibitors.

A major limitation in using crude testicular plasma membranes for detergent solubilization has been the lower recovery of solubilized FSH receptors (31, 32), requiring purification of membrane-containing fractions prior to detergent extraction. Sucrose density gradient centrifugation offers the most straightforward and efficient method to purify crude plasma membranes. Use of a Ti-15 rotor of 1.6-liter capacity in the present study facilitated a preparative fractionation of concentrated 7000 × g supernatant by sucrose density gradient centrifugation. Significant amounts of lipid and inactive proteins (85%) were separated from FSH receptor-containing fractions by this procedure. Moreover, a side fraction (SDG Fraction III) isolated during centrifugation appeared to be a potential starting fraction for isolation of LH/hCG receptor from testis.

Perhaps the greatest obstacle to the isolation of follitropin receptors is the identification of procedures which disrupt the membrane lipid matrix and extract receptors without causing denaturation. A series of systematic studies indicated that an optimum ratio of protein to Triton X-100 is of critical importance for the extraction of stable, active FSH binding activity (13). The optimum ratio of protein to Triton X-100 was found to be 20 mg of protein to 1 ml of 0.2% Triton X-100. An interesting feature of bovine testis FSH receptor solubilized by Triton X-100 under these conditions was its slight, but reproducibly higher, FSH-binding activity (1.4-fold) than that of membrane-bound receptor. Increased hormone-binding activity following membrane solubilization has also been reported for prolactin and thyrotropin receptors (33, 34).

The exposure of membranes to organic solvents under conditions which extract lipids or perturb the internal arrangements of lipids can facilitate the extraction of membrane proteins (35, 36). Petroleum ether was previously shown to be effective to facilitate the solubilization of LH/hCG receptors from bovine (22) as well as rat (37) ovarian membranes. In the present study, a simultaneous treatment of the Triton X-100-solubilized fraction from DC-2 concentrate with 0.2 volume (v/v) of petroleum ether removed free lipids in the organic phase and resulted in a 40% increase in the FSH-binding activity compared to untreated membranes. The detergent-soluble fraction from DC-2 concentrate after centrifugation at 145,000 × g retained full specific FSH-binding activity, while hCG-binding activity was only 47% of that originally present. Approximately 88% of the follitropin receptors originally present in the light membranes were extracted by detergent under the above optimum conditions, and this is a considerable improvement over the 20–40% recovery obtained in the previous reports (31, 32). Importantly, the soluble follitropin receptors in the detergent extract were stable and functional as reflected by FSH stimulation of adenylate cyclase (13) suggesting that the fraction could be an ideal precursor for further purification of receptor.

Since follitropin receptor after chromatography on Sepharose 6B was a complex of receptor and G-protein (3), we examined the conditions under which the complex could be dissociated. Detergents at sufficiently elevated concentrations are known to uncouple the functional units of adenylate cyclase (28, 29). We, therefore, exposed 6B-Fr-1 to a higher concentration of Triton X-100, while maintaining the optimum ratio between sample protein and Triton previously shown to preserve FSH receptor activity (13). This was achieved through a 2.5-fold concentration of 6B-Fr-1 by ultrafiltration using an Amicon TCF-2 unit fitted with a Diaflow YM-30 membrane (exclusion limit M, < 30,000), without loss of receptor activity. Excessive concentration of active material should be avoided as this leads to sample turbidity with loss of FSH-binding activity.

Through an application of ion exchange chromatography on DEAE-cellulose, an excellent separation between FSH receptor and Gpp(NH)p binding activities was achieved. One of the common features of the Gpp(NH)p-binding protein, LH/hCG receptors, is that they are effectively adsorbed by anion exchange resin (22, 40, 41). Clearly, the Gpp(NH)p binding activity and low (but significant) LH/hCG receptors present in 4B-Fr-2 as contaminants were adsorbed by DEAE-cellulose and eluted, which is consistent with the findings from previous reports (22, 40, 41). Most FSH-binding activity of 4B-Fr-2 was unadsorbed by DEAE-cellulose as described under "Materials and Methods," resulting in a significant purification of 4B-Fr-2 fraction.

Like most cell surface proteins, membrane receptors generally contain covalently bound carbohydrate chains (42–45). Most FSH receptor activity present in the DEAE unadsorbed fraction was adsorbed by wheat germ lectin and eluted by the specific sugar, N-acetylglucosamine, indicating a glycoprotein nature of FSH receptor. A common feature of receptors for FSH, LH/hCG (44, 45), and TSH (43) appears to be their glycoprotein nature. Another advantage of lectin affinity chromatography was the separation of trace amounts of Gpp(NH)p binding activity and inactive proteins of nonglycoprotein nature from FSH receptor. The final receptor preparation was free of [3H]-hCG binding and Gpp(NH)p binding activities, and was stable when stored in buffer containing 0.02% Triton X-100 and 30% glycerol at 1–4 °C for 1 week or several months at −80 °C.

Due to limited amounts of starting material, and decreased or loss of activity of soluble FSH receptor reported in earlier studies (11, 12), the yields in those studies cannot be compared with those from the current study. From the 24,750 pmol of receptor activity present in 25 liters of 7,000 × g supernatant, 1,823 pmol of receptor activity was recovered in 526 μg of protein of receptor, representing a 7.4% yield from 7,000 × g supernatant and 85% yield from detergent-soluble petroleum ether-treated fractions (Table 1). Several lines of evidence established the purity of the final receptor preparation. Upon SDS-polyacrylamide gel electrophoresis, the preparation gave a single band in the region of M, ~ 240,000 under nonreduced and M, ~ 60,000 under reduced conditions. The receptor preparation could be effectively adsorbed to the ovine FSH Affi-Gel 10 column. An elution of bound follitropin receptor activity yielded an active preparation upon SDS-PAGE analysis and under reduced conditions gave a major band in the region of M, 60,000 (not shown). The affinity cross-linking of the purified receptor to [3H]-hFSH produced a complex which, upon SDS-PAGE, revealed a band in the region of 300,000 under nonreducing conditions. The band, however, was abolished after incubation.
FSH Receptor of Bovine Calf Testis

TABLE I

Yield and specific activity of receptor-containing fractions isolated during purification of follitropin receptor from several batches of bovine calf testes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein recovery</th>
<th>FSH binding</th>
<th>Total FSH bound</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x 10^-15 M</td>
<td>Specific activity</td>
<td>pmol</td>
<td>%</td>
</tr>
<tr>
<td>Testes homogenate</td>
<td>492.0 ± 68^</td>
<td>0.51</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>7,000 x g supernatant</td>
<td>198.0 ± 19</td>
<td>0.74</td>
<td>125</td>
<td>24,750</td>
</tr>
<tr>
<td>7,000 x g supernatant concentrate</td>
<td>125.0 ± 16</td>
<td>0.48</td>
<td>161</td>
<td>90,125</td>
</tr>
<tr>
<td>Sucrose density gradient Fr-11</td>
<td>5.05 ± 0.52</td>
<td>0.98</td>
<td>814</td>
<td>4,111</td>
</tr>
<tr>
<td>DC-2 concentrate</td>
<td>3.86 ± 0.39</td>
<td>0.72</td>
<td>823</td>
<td>3,176</td>
</tr>
<tr>
<td>Triton-solubilized, petroleum ether-treated 6B-Fr-1</td>
<td>2.4 ± 0.22</td>
<td>0.46</td>
<td>1,100</td>
<td>2,700</td>
</tr>
<tr>
<td>4B-Fr-2</td>
<td>0.0619 ± 0.011</td>
<td>1.80</td>
<td>5,230</td>
<td>2,179</td>
</tr>
<tr>
<td>DE-Fr-1 &amp; 2</td>
<td>0.00199 ± 0.00026</td>
<td>1.95</td>
<td>27,512</td>
<td>1,703</td>
</tr>
<tr>
<td>Wheat germ agglutinin-Fr</td>
<td>0.000528 ± 0.00006</td>
<td>3.176</td>
<td>3,466,667</td>
<td>1,823</td>
</tr>
</tbody>
</table>

* Preparation also contains low affinity and high capacity FSH-binding sites (0.71 x 10^-4 M).

* Preparation also contains low affinity and high capacity FSH-binding sites (0.71 x 10^-4 M).

of the receptor with 125I-hFSH in the presence of excess unlabeled FSH. A polyclonal antibody raised against the receptor preparation specifically inhibited the binding of 125I-hFSH to membrane-bound FSH receptors (46). Also, the antibody behaved as an agonist causing increased conversion of androstenedione to estradiol in rat Sertoli cells (46). These findings provide immunological evidence for receptor specificity of the purified preparations. The fact that FSH preparations from different species effectively competed with radioiodinated hFSH for binding to purified receptor, while heterogenous hormones such as LH/hCG, or bovine TSH failed to do so, indicated a high degree of hormone specificity, but lack of species specificity among the follitropin interactions with the purified receptor.

Our results on the structure of the purified FSH receptor, as well as those from previous studies on photoaffinity (7-10) and chemical cross-linking (7) of hormone to membrane-bound receptor, revealed an oligomeric nature of the follitropin receptor. The photoaffinity cross-linking studies by Smith et al. (8, 9) have suggested that FSH receptor of bovine calf testis consists of at least three subunits of M, 32,000, 48,000, and 86,000, respectively. In contrast, the FSH receptor of porcine granulosa cells was shown to contain three subunits of markedly different size (M, 18,000, 22,000, and 34,000). Moreover, the chemical crosslinking studies have shown that calf testis FSH receptor contains subunits of M, 45,000 or 49,000 (7). It may be emphasized that endogenous proteolysis could produce receptor-derived proteolytic fragments and increased multiplicity that could lead to erroneous interpretation of receptor structure. Moreover, varying amounts of detergent binding, hormone binding, and covalent coupling of the hormone to the receptor could significantly affect the conformation and thereby the Stokes radii of the molecules (38). In the present study, the purified FSH receptor behaved as a single entity of M, ~240,000 under nonreducing conditions, and was not affected by treatment with urea (8 M), suggesting that the receptor may not contain noncovalently associated subunits. Treatment with the reducing agent di-thiothreitol induced loss of the 240,000 band, with the appearance of a band with M, ~60,000. Since the 60-kDa band did not dissociate further in the presence of higher concentrations of di-thiothreitol (200 mM) and did not further resolve upon SDS-PAGE in a higher percent acrylamide gel, the possibility that this band (~60 kDa) is a receptor subunit seems plausible. Moreover, when the receptor-hormone complex was chemically cross-linked and analyzed by SDS-PAGE under reduced conditions, it showed a radioactive band of M, ~88,000 suggesting that the 60,000 component was specifically labeled by 125I-hFSH (50). A polyclonal antireceptor antibody raised in rabbits immunoprecipitated the two forms of FSH receptor under nonreduced and reduced conditions (51). Furthermore, both 240- and 60-kDa bands gave identical patterns of peptides after partial proteolytic digestion.

The oligomeric nature of the receptors for glycoprotein hormones such as LH/hCG and TSH is noteworthy (22, 43, 47, 48) and has raised conflicting opinions. It has been suggested that the formation of oligomeric form during purification seems to be unique to LH/hCG receptors and is probably caused by the intermolecular oxidation of the free—SH groups present in the receptor to form disulfide bonds (49). Other studies, however, have suggested that the native LH/hCG receptor is a homodimer and its functional form may, in fact, be a homodimer of the 90-kDa species (44). As regards to the evidence on follitropin receptor structure, it is important to emphasize that further investigations are needed to determine the functional form of the follitropin receptor in the Sertoli cell plasma membrane. Finally, the availability of follitropin receptor in a highly purified and stable state should facilitate studies on its molecular biology and structure-function relationships.

REFERENCES

FSH Receptor of Bovine Calf Testes

Briefly, the FSH-C receptor (FSH-C) was purified from calf testes and used for receptor binding studies. FSH-C receptor binding was measured by the equilibrium dialysis technique with multiple disulfide bonds, and the specificity of FSH-C receptor binding was determined by competition with various ligands. Results were analyzed by Scatchard analysis, and the dissociation constant (Kd) of FSH-C receptor binding was determined.

**Methods**

**Receptor Binding Studies**

**Materials and Methods**

**Results**

**Discussion**

**Conclusion**

**Acknowledgments**

**References**

**Supplementary Material**

**Appendix**

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**Analytical Methods**

**Protein estimation**

The protein content of the testicular homogenate, spermatozoa preparations or column eluates was determined by the method of Lowry et al. (1951). A standard curve was prepared using bovine serum albumin as the standard.

**Receptor-binding assay**

Highly purified bovine FSH (LHRH 1749-1750) and HCG (CH 119) were radiolabeled using the lactoperoxidase method (15) with some modifications (11). We used an elution buffer containing 10 mM Tris-Cl buffer, pH 7.2, 0.1% 2-mercaptoethanol, 10% glycerol, and 0.25% BSA. The receptor-binding assay was performed using a Scatchard analysis (13).

**Characterization of the FSH-Receptor Complex**

The receptor-binding capacity of the bovine testis was determined by competition with various ligands. Results were analyzed by Scatchard analysis, and the dissociation constant (Kd) of FSH-C receptor binding was determined.

**References**

Molecular assembly and oligomeric nature of the folliculin receptor—
molecularly purified receptor. FSH-R-150 was reconstituted as described above and aliquots of the radiolabeled protein were treated separately as follows:

a) Two sample aliquots were incubated in 4 x 6 M NaCl (final), for 4 h at 30°C. The treated samples were then assayed by autoradiography.

b) A third sample aliquot was incubated in 28 SDS (final) for 90 min at 30°C and analyzed by PAGE-PAGE.

c) Other sample aliquots were incubated in 28 SDS (final) and 1, 3, 5, 7, or 20 AM GTT (final) for 90 min at 30°C and analyzed by PAGE-PAGE.

The results of these experiments revealed higher activity of both complexes, with significant differences in the molecular weight of the radiolabeled protein compared to untreated membranes. The amino acid content was further confirmed at 150 min and 90 min. The soluble receptor fraction (supernatant) contained a large protein with a full retention of specific 125I-FSH-binding activity.

Enzymatic digestion of receptor/substrates— Purified receptor and substrate were incubated at pH 5.0 and adjusted to the digestion concentration to the substrate and the final concentration of 0.1 M NaCl and 60 mM KCl were added, followed by incubation in the sample wells of a second SDS gel (15% acrylamide) and each gel overlayed with a final concentration of 80 μM of a 32P-labeled nucleoside triphosphate. Digestion proceeded inside the stacking gel during subsequent electrophoresis. After electrophoresis, the gels were dried prior to autoradiography at -80°C.

RESULTS

Preparative centrifugation and concentration of the FSH sубmг protein from bovine calf testis— In each batch, 13.1 kg of bovine calf testis (fresh) were decapsulated and an average wet tissue weight of 6000 g was homogenized as described in Materials to obtain a total tissue homogenate of 20 liters containing an average protein yield of 493 g with a specific activity of 610 μg/ml of protein. After centrifugation at 10,000 x g for 20 minutes, 70% of the supernatant containing an average of 19.8 g of protein with a specific activity of 56.8 IU/μg of protein was concentrated and stored at liquid nitrogen temperature. High affinity and high capacity FSH-binding sites were removed to 700,000 x g sediment. The detailed characteristics of these fractions have been previously reported [11]. A 20-fold concentration of 7,000,000 x g supernatant by isoelectric focusing, using a Phoscol-10 column and a hollow-fiber cellulose acetate-50 (HAC) with an elution limit of 50,000 Da, resulted in an average protein recovery of 5% with a specific activity of 181 fmoI/mg protein.

Purification of testicular light phase membrane— A total of 1.1 liter of concentrated 7,000 x g supernatant, obtained from a 15.1 kg batch of testes was processed in ten sucrose density gradient (10-30%) centrifugations, using a large capacity zonal rotor [17-19]. Significant amounts of 125I-labeled phosphorylated and non-phosphorylated FSH-binding activity was added and used as 125I-FSH-binding activity. Significant FSH-binding activity was also added and used as 125I-FSH-binding activity. Significant 125I-FSH-binding activity was also added and used as 125I-FSH-binding activity.

Fig. 1: Preparative centrifugation in linear sucrose density gradient of concentrated 7,000 x g supernatant. Approximately 12.5 g of protein was layered over an NCS sucrose cushion containing 12% sucrose, 2.5 M sucrose solution and 2.5 M sucrose solution. The fractions were pooled into three major fractions (120 fractions 1, 1111) and injected by arrows in the figure based on protein content and receptor activity of individual fractions.

Fig. 2: A flow chart of a typical procedure developed for the purification of folliculin receptor from detergent-soluble fraction of calf testis to lighter membrane.

Fig. 3: SDS-agarose gel electrophoresis of purified receptor, untreated protein, digested with or without 125I-FSH-binding activity. Significant 125I-FSH-binding activity was also added and used as 125I-FSH-binding activity. Significant 125I-FSH-binding activity was also added and used as 125I-FSH-binding activity.
Fig. 3: Elution profiles of FSH receptor and \(^{3}H\)Gpp(NH)p-binding activity after chromatography on Sepharose 4B. Concentrated Fr-1 of 0.3 mg protein was applied on a Sepharose 4B gel bed of 38 x 5 cm. The column was equilibrated and eluted with 10 mM Tris-HCl buffer, pH 7.2, containing 1 mM MgCl\(_2\), 0.001% NaN\(_3\), 5% glycerol and 0.0375% Triton X-100, at a flow rate of 15 ml/hr. Individual eluents were analyzed for \(^{3}H\)Gpp(NH)p-binding activity (A). Eluted samples were pooled into three major fractions: (Fr-1), Fr-2 and Fr-3, as indicated by arrows in the figure.

DEAE-cellulose chromatography: Approximately 12.7 mg protein aliquot of Sepharose 4B-Fr-2 was further purified each time by ion exchange chromatography. As shown in figure 4, most G-protein-binding, Gpp(NH)p-binding activities, and residual \(^{3}H\)nucleotide binding activity present in Fr-2-Fr-3 were adsorbed by DEAE-cellulose and could be eluted by 0.1 M ammonium acetate, pH 7.5 (Fr-3-2) and 0.2 M ammonium acetate, pH 6.5 (Fr-3-3). The FSH-binding activity of Fr-2-Fr-3, however, was adsorbed by DEAE-cellulose and could be eluted in breakthrough (Fr-3-1) and buffer wash (Fr-3-2) fractions. The average recovery of protein in these fractions was 59% of total protein. Fractions Fr-3-2 and Fr-3-3 contained high affinity follitropin receptors (Table 1) with specific activity 36-fold higher than in Fr-2-Fr-3. The Gpp(NH)p-binding activity in Fr-3-1 and Fr-3-3 was extremely low, and an excellent separation between the two activities was achieved by DEAE-cellulose chromatography (Fig. 4).

Lectin affinity chromatography: - Fractions Fr-3-2 and Fr-3-3 were pooled and further purified by affinity chromatography using agaro-linked wheat germ agglutinin (WGA), a lectin that specifically binds to N-acetyl glucosamine and sialic acid residues. As shown in figure 5, greater than 80% of the total FSH receptor activity present in fractions Fr-3-2 and Fr-3-3 was adsorbed by immobilized lectin, and could be eluted with 0.2 M N-acetyl glucosamine (WGA-Fr-3) and 2.4 M N-acetyl glucosamine (WGA-Fr-4). These amounts of Gpp(NH)p-binding activity present in the applied sample were unabsorbed by the lectin. The resulting fractions (WGA-Fr-3) and WGA-Fr-4) contained purified high affinity follitropin receptors (Table 1), with an average FSH-binding capacity of 3.47 (1.7-4.3) pmol/mg protein, which corresponds to 0.02 µg of FSH bound per mg of receptor (249 kDa).

Yield and activity of the follitropin receptor: Table 1 shows the composite data on recovery of FSH receptor activity and increase in specific FSH-binding activity of the fractions isolated from several batches (11.5 kg) of calf testes. 94% recovery of protein indicates 7.6-fold enrichment. A 44% recovery of protein indicates 9.3-fold enrichment. As the fractions isolated from several batches of calf testes were concentrated by 0.2 M ammonium acetate, the yield of the FSH receptor in the purified receptor preparation was 60% of the total FSH receptor protein recovered from the testes. The specific activity of the purified receptor preparation was 0.15 pmol/mg of protein. When stored in 20 mM Tris-HCl buffer, pH 7.5, containing 10% DMSO, 0.01% NaN\(_3\), 0.025% Triton X-100 and 50% glycerol, was stable for at least 6 months at -80°C, or for a week at 4°C.
Characterization of the Follitropin Receptor

Nonpolyacrylamide gel electrophoresis. The purified fraction obtained after lectin affinity chromatography, when treated with 24 bp alone for 90 s at 100°C, gave a single band with \( R_1 = 240600 \) as compared to 25-30 bands stained by the light sensitive protein fraction, indicating a high degree of purity and electrophoretic homogeneity of the purified receptor (Fig. 6). After treatment with 7M, M dimethylsulfoxide (DMSO) and 20 M for 90 s at 100°C, the purified receptor showed a single minor stained band with \( R_2 = 65600 \) (Fig. 6).

Nonpolyacrylamide gel electrophoresis analysis of the \(^{125}\)I-FSH-binding data revealed the presence of only a single class of high affinity FSH-binding sites in all active fractions obtained during various stages of purification (Table 1). The dissociation constants \( K_d \) of receptors present in deglycosylated tetractin fraction, monomer 66 (fraction 1), and WGA fraction 230 were \( 4.6 \times 10^{-14} \)M, \( 3.8 \times 10^{-14} \)M, and \( 3.9 \times 10^{-14} \)M (Fig. 1), respectively, suggesting that there was only a small change in the affinity of the receptor during purification. The high affinity, low capacity binding sites in the highly purified follitropin receptor ranged from \( 1.7 \times 10^{-16} \) M for 99% of the receptor.

Specificity of \(^{125}\)I-FSH-binding to the purified receptor preparations. A major discovery by which receptors are characterized is their specificity for binding known ligands. As shown in figure 2, unlabeled FSH, hCG, and hCG effectively inhibited binding of \(^{125}\)I-FSH to purified receptor in a concentration-dependent manner. Similar activity of hCG and hCG did not inhibit \(^{125}\)I-FSH binding at concentrations up to 50 \( \mu \)g (BLR, BSRM) or 25 \( \mu \)g (HCG). These results substantiate a high degree of hormone specificity as well as absence of cross-reactivity in FSH interaction with the purified receptor.

Specificity of \(^{125}\)I-FSH-binding to the purified receptor preparations. Aligants (7.5 \( \mu \)g of protein) were incubated with \(^{125}\)I-FSH at \( 4^\circ \)C for 1 h at 4°C, together with indicated concentrations of unlabeled FSH ( ), hCG ( ), d-FSH ( ), and hCG ( ). Each point represents the mean of triplicate measurements.

Fig. 6: SDS-polyacrylamide gel electrophoresis of purified follitropin receptor. Panel A, schematic-dimension light sensitive protein fraction (93,550 daltons), active fraction (43,500 daltons) after lectin affinity chromatography. 24 bp. Samples were reconstituted in 0.24 M Tris-HCl buffer, pH 7.4, containing 27 2000# alone (Panel A, lane 1) or 27 2000# and 20 M dimethylsulfoxide (Panel A, lane 3) and heated in boiling water for 90 s, and then electrophoresed in 4% (Panel A) and 10% (Panel B) gels at 75 and 100 V, respectively, with an anode in the middle. The wells were silver stained. The positions of the \( R_2 \) standards are indicated by arrows in the figure.

Fig. 7: Scatchard plot for the binding of \(^{125}\)I-FSH to purified FSH receptor. Purified receptor was incubated with a constant amount of \(^{125}\)I-FSH and increasing amounts of unlabeled FSH as described under METHODS. The data were expressed in the form of Scatchard plot.

Fig. 8: Specificity of \(^{125}\)I-FSH-binding to the purified receptor preparations. Aligants (7.5 \( \mu \)g of protein) were incubated with \(^{125}\)I-FSH at \( 4^\circ \)C for 1 h at 4°C, together with indicated concentrations of unlabeled FSH ( ), hCG ( ), d-FSH ( ), and hCG ( ). Each point represents the mean of triplicate measurements.
The oligomeric nature of the FSH receptor - Lectin affinity purified FSH receptor preparation was radioiodinated to specific activities of 10 - 62 
Ci/mg. Following treatment of the receptor with 0.5 M for 2 h at 0°C, and 
analysis by SDS-PAGE and autoradiography, a major band with an apparent Mₚ of 66,000 was observed (Fig. 9A). Occasionally, an additional first band 
corresponding to Mₚ of 170,000 was also noted (Fig. 9B). This component, 
however, was unobserved by an ANTI-FLCH IgG affinity column, whereas the 
receptor was absorbed and could be eluted by 50 mM sodium acetate buffer, pH 
5.5, containing 1 mM EDTA (not shown). Treatment of radioiodinated receptor with 
4 or 8 h urea for 2 h at 37°C, prior to 2DE-PAGE, did not alter the molecular 
weight (Mₚ ~ 240,000) of the receptor (Fig. 12) suggesting that the receptor 
does not contain non-covalently associated subunits. However, the treatment 
with 24 &D for 8 h at 37°C, followed by dialysis and then treatment with DTT (1 to 70 mM) for 45 s (at 
15°C) induced a loss of Mₚ ~ 240,000 band with appearance of an Mₚ ~ 66,000 
band (Fig. 9C). Exposure of the complex preparation to 200 mM of DTT did 
not further dissociate the Mₚ ~ 66,000 band. Moreover, the band with Mₚ of 
65,000 identified in 7.5% acrylamide gel under reduced conditions, gave a 
single band following electrophoresis in the second dimension in 15% 
acrylamide gels (Fig. 11). Also, the purified receptor was incubated with 
125I-iodo in the presence and absence of unlabeled FSH and the hormone- 
receptor complexes were extensively cross-linked with DSS. Analysis of cross-
linked complexes by SDS-PAGE under non-reducing conditions followed 
by autoradiography revealed a single prominent band in the region of Mₚ ~ 315,000 
(Fig. 12). To further understand the relationship between 240,000 and 66,000 
bands of receptor, we have subjected both radiative bands to peptide 
mapping by limited proteolysis by Staphylococcal proteinase 109 pretreatment. SDS-PAGE analysis (Fig. 13) revealed that the pattern of the proteins produced after 
partial proteolytic cleavage, were identical for both 240 kDa and 60 kDa bands.

Fig. 9: non-polyacrylamide gel electrophoresis analysis of purified 125I-
iodinated receptor, under non-reduced and reduced conditions. Noniodinated 
receptor preparation was taken in 0.025 M Tris-Cl buffer, pH 7.4, containing 
1% SDS and heated in boiling water for 90 s. The sample, after dialysis 
against 0.025 M Tris-Cl buffer, pH 7.4, containing 2.3% SDS was either 
directly electrophoresed in 6% acrylamide separating gel (Panel A) or 
incubated for 45 s in the presence of 0, 1, 5, 15, 70 or 200 mM DTT and then 
electrophoresed in 7.5% acrylamide separating gel (Panel B, lanes 1-6). Gels 
were dried before autoradiography. The positions of Mₚ standards are 
indicated by arrows in the figure.

Fig. 10: SDS-polyacrylamide gel electrophoresis analysis of purified 125I-
iodinated FSH receptor before and after treatment with urea. Purified 125I-
iodinated receptor preparation was incubated for 4 h at 17°C in the absence or 
presence of indicated concentrations urea. The aliquots treated and 
electrophoresed by SDS-PAGE, under non-reducing conditions in 7.5% acrylamide 
separating gel were as described in the legend for figure 9. Panel B, lane 1. 
Gels were stained with Coomassie blue and dried before autoradiography. The 
positions of Mₚ standards are indicated by arrows in the figure.

Fig. 11: Anti-electrophoresis of radiolabeled receptor component (~40 kDa band) 
in the second dimension (SDS-PAGE). After the first-dimension SDS-PAGE, under 
non-reduced conditions (70 mM DTT) as described in the legend of figure 9, Panel 
B, the lane containing ~40 kDa band was cut and placed horizontally over a 
fresh 15% acrylamide separating gel for electrophoresis. Electrophoresis 
followed by autoradiography of gel was carried out as described in REMODE.
Fig. 12: Covalent cross-linking of 125I-FSH to purified FSH receptor. Unlabeled receptor after SDS-PAGE chromatography was incubated with 125I-FSH in the absence (lane 1) or presence of excess unlabeled FSH (lane 2). The hormone-receptor complexes were separated free from unbound 125I-FSH by gel filtration, and cross-linked by the bifunctional reagent dimethylsuberimidate, as described in METHODS. The covalently cross-linked hormone-receptor complexes were analyzed by SDS-PAGE under non-reduced conditions in an acrylamide separation gel containing 3.1% SDS, followed by autoradiography. The positions of standard proteins are indicated by arrows in the figure.

Fig. 13: Peptide maps of purified receptor and subunits after digestion with Staphylococcus aureus V8 protease. Electrophoresis of 125I-receptor was first performed (see Fig. 9, Panel B). The gel slices of interest (240 km and 40 km bands) were sliced, inserted in the sample wells (lane 1 and lane 2, respectively) of a second SDS gel (15% acrylamide) and overlaid with gel with Staphylococcus aureus V8 protease (final concentration of 80 μg/ml). Electrophoresis was performed in the usual manner with the exception that the current was turned off for 30 min when the bromophenol blue dye in the control well reached the bottom of the stacking gel. Dissection proceeded directly in the stacking gel. An autoradiogram of a dried gel is shown.
Purification of follitropin receptor from bovine calf testes.
B Dattatreyamurty, S B Zhang and L E Reichert, Jr


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