Purification of Follitropin Receptor from Bovine Calf Testes*

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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 \( 1.9 \times 10^{-16} \text{ M} \). Yield was 526 \( \mu \text{g/11.5 kg testes} \). Radiiodinated, as well as unlabeled purified FSH receptor, migrated on sodium dodecyl sulfate-polyacrylamide gels as a single major band of \( M_r = 240,000 \). This band was not affected by 8 \( M \) urea prior to analysis. The detergent-soluble fraction contained stable and functional FSH receptors. The approach is dependent on isolation of receptor-enriched light membrane fractions from bovine 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. The resulting detergent-soluble fraction contained stable and functional follitropin receptors and represented an ideal precursor for its further purification. We report here the purification of the follitropin receptor from bovine testes.

MATERIALS AND METHODS AND RESULTS

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 testes 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 7000 \( \times g \) sediment fraction containing heavier plasma membranes. The 7000 \( \times g \) 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 membrane 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 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. 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 testes.

DISCUSSION

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 pro-
cessed.

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 recep-
tor-binding inhibitors (28, 29). These inhibitors, if not re-
moved, 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 7000 x g supernatant by Amicon DC-10 ultrafiltration
was carried out in the presence of a protease inhibitor, sodium
p-hydroxymercuribenzoate. This rendered FSH receptor ac-
tivity in the resulting concentrate more stable and also re-
moved significant amounts of inactive proteins as well as FSH
receptor-binding inhibitors.

A major limitation in using crude testicular plasma mem-
bryons for detergent solubilization has been the lower recovery
of solubilized FSH receptors (31, 32), requiring purification
of membrane-containing fractions prior to detergent extrac-
tion. 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 con-
centrated 7000 x g supernatant by sucrose density gradient
centrifugation. Significant amounts of lipid and inactive pro-
teins (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 impor-
tance 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 ac-
tivity following membrane solubilization has also been re-
ported for prolactin and thyrotropin receptors (33, 34).

The exposure of membranes to organic solvents under
conditions which extract lipids or perturb the internal ar-
rangements 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 recep-
tors 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 de-
tergent-soluble fraction from DC-2 concentrate after centri-
fugation at 145,000 x 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% recov-
ery obtained in the previous reports (31, 32). Impor-
tantly, the soluble follitropin receptors in the detergent ex-
tact were stable and functional as reflected by FSH stimu-
lation of adenylate cyclase (13) suggesting that the fraction
could be an ideal precursor for further purification of receptor.

Since follitropin receptor after chromatography on Sepha-
rose 6B was a complex of receptor and G-protein (3), we ex-
amined the conditions under which the complex could be
dissociated. Detergents at sufficiently elevated concentrations
are known to uncouple the functional units of adenylate
cyclase (38, 39). We, therefore, exposed 6B-Fr-1 to a higher
concentration of Triton X-100, while maintaining the opti-
mum 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 ul-
trafiltration using an Amicon TCF-2 unit fitted with a Diaflow
YM-30 membrane (exclusion limit M, < 30,000), without loss
of receptor binding 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
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 gen-

erally 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-acetylgalactosamine, indicating a glycoprotein
nature of FSH receptor. A common feature of receptors for
FSH, LH/hCG (44, 46), and TSH (45) 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 nongly-
coprotein nature from FSH receptor. The final receptor pre-
paration 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 x g supernatant,
1,823 pmol of receptor activity was recovered in 526 pg of
protein of receptor, representing a 7.4% yield from 7,000 x g
centrifugation. Significant amounts of lipid and inactive pro-
teins of receptor 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 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 (38, 39). We, therefore, exposed 6B-Fr-1 to a higher
concentration of Triton X-100, while maintaining the opti-
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YM-30 membrane (exclusion limit M, < 30,000), without loss
of receptor binding activity. Excessive concentration of active
material should be avoided as this leads to sample turbidity with
loss of FSH-binding activity.

Several lines of evidence established the purity of the final
receptor preparation. Upon SDS-polyacrylamide gel electo-
rophoresis, 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 effec-
tively 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, re-
vealed a band in the region of 300,000 under nonreducing
conditions. The band, however, was abolished after incubation
of the receptor with $^{125}$I-hFSH in the presence of excess unlabeled FSH. A polyclonal antibody raised against the receptor preparation specifically inhibited the binding of $^{125}$I-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 immunochemical evidence for receptor specificity of the purified preparations. The fact that FSH preparations from different species effectively competed with radiiodinated hFSH for binding to purified receptor, while heterogenous hormones such as bLH, hCG, or bovine TSH failed to do so, indicated a high degree of hormone specificity, but lack of species specificity among the follitropin interac-
tions 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 $M_e$ 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 (Mr 18,000, 22,000, and 34,000). The calf testis FSH receptor contains subunits of Mr 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 interpreta-
tion 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 Mr ~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-
raithiocthreitol induced loss of the 240,000 band, with the op-
perience of a band with Mr ~ 60,000. Since the 60-kDa band did not dissociate further in the presence of higher concentrations of diathiocthreitol (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 Mr ~ 88,000 suggesting that the 60,000 component was specifically labeled by $^{125}$I-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, 44, 47, 48) and has raised conflicting opinions. It has been suggested that the formation of oligomeric form during puri-
fication 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-func-
tion relationships.

#### REFERENCES


#### TABLE I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein recovery</th>
<th>FSH binding</th>
<th>Total FSH bound</th>
<th>Recovery</th>
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<tr>
<td></td>
<td>%</td>
<td>$K_a$, pM</td>
<td>Specific activity</td>
<td>%</td>
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<tr>
<td>Testes homogenate*</td>
<td>492.0 ± 68</td>
<td>0.51</td>
<td>48</td>
<td></td>
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<td>7,000 × g supernatant</td>
<td>198.0 ± 19 (10)</td>
<td>0.74</td>
<td>125</td>
<td>2,701</td>
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<tr>
<td>7,000 × g supernatant conc</td>
<td>125.0 ± 16 (10)</td>
<td>0.48</td>
<td>161</td>
<td>3,176</td>
</tr>
<tr>
<td>Sucrose density gradient Fr-11</td>
<td>5.05 ± 0.52 (10)</td>
<td>0.98</td>
<td>814</td>
<td>11.3</td>
</tr>
<tr>
<td>DC-2 concentrate</td>
<td>3.86 ± 0.39 (10)</td>
<td>0.72</td>
<td>823</td>
<td>12.8</td>
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<tr>
<td>Triton-X100 solubilized, petroleum ether-treated</td>
<td>2.4 ± 0.22 (6)</td>
<td>0.46</td>
<td>1,100</td>
<td>11.3</td>
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<tr>
<td>6B-Fr-1</td>
<td>0.415 ± 0.03 (4)</td>
<td>1.10</td>
<td>5,230</td>
<td>8.8</td>
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<tr>
<td>4B-Fr-2</td>
<td>0.0619 ± 0.011 (4)</td>
<td>1.95</td>
<td>27,512</td>
<td>6.9</td>
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<tr>
<td>DE-Fr-1 &amp; 2</td>
<td>0.00199 ± 0.00026 (4)</td>
<td>990,476</td>
<td>1,991</td>
<td>0.8</td>
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<tr>
<td>Wheat germ agglutinin-Fr</td>
<td>0.000526 ± 0.00006 (3)</td>
<td>1.90</td>
<td>3,466,667</td>
<td>7.4</td>
</tr>
</tbody>
</table>

* Preparation also contains low affinity and high capacity FSH-binding sites ($0.71 \times 10^{-5}$ M).

* From 11.8 kg of bovine calf testes. Values are means ± S.D. for number of observations in parentheses.
FSH Receptor of Bovine Calf Testis

Supplemental Material to

Purification and Primary Structure of Polliotin Receptor from Bovine Calf Testis

Housakos Dattatreyaamurty, Shao-Bin Zhang, and Lee E. Reichert, Jr.

MATERIALS

Calf testes were obtained from a local abattoir and kept at -20°C until use. Lactoperoxidase, polyethylene glycol (PEG 4000), 0.1M-tricethanolamine, 0.01M-Tris-HCl buffer (pH 8.4), 0.1M-NaCl, 0.1M-MgCl₂, bovine serum albumin, and all other chemicals were purchased from Sigma Chemical Co., St. Louis, MO. [3H]NAGP was from ICN Biochemicals, Irvine, CA. [3H]- and [125I]-labeled FSH were purchased from the National Pituitary Agency, Bethesda, MD. [3H]NAGP was purchased from Amerham Chemical Company, Milwaukee, WI. Diisopropylfluorophosphate was obtained from Pierce, Rockford, IL. Sephadex G-25, Sephadex G-50, and amphotericin B gent in was purchased from Pharmacia-LKB, Piscataway, NJ. TPC-T-2 ultrafiltration unit and dialysis tubing were purchased from Amicon, Beverly, MA. Sephadex G-25 was obtained from Pharmacia-LKB, Piscataway, NJ. MTT assay was purchased from BioMerieux-Norwalk, Norwalk, CT. 

METHODS

Purification of the follitropin receptor

Frozen calf testes in batches of 15 kg were thawed, degased, and homogenized in Tris-HCl buffer (100 mM Tris-HCl, pH 7.4, containing 1 mM MgCl₂, 10 mM p-hydroxymercuribenzoate, 0.001% mercaptoethanol, 1% Tween-80, and a 7.0 ug x g of bovine per 1g of testis, containing 1 g of 10 mg of testis). 

Abolishment of the follitropin receptor in Triton X-100 and removal of the free ligands by interaction with specific antibodies.

Follitropin receptor from light chains were abolished by Triton X-100 under optimum conditions, according to a procedure previously described by us (13).
FSH Receptor of Bovine Calf Testis

The FSH receptor is a G-protein-coupled receptor that mediates the action of follicle-stimulating hormone (FSH) on the testis. It is expressed in Leydig cells, where it regulates steroidogenesis. The FSH receptor is a transmembrane protein that consists of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular signaling domain.

Analytical Methods

Protein estimation: The protein content of the testicular homogenate or column effluent fractions was determined by the method of Lowry et al. (1). The bicinchoninic acid (BCA) protein assay kit (Pierce) was used for the determination of protein concentrations.

Characterization of the FSH Receptor

Cross-linking experiments: The cross-linking of the FSH receptor with a disulfide catalyst was performed to study the quaternary structure of the receptor. The cross-linker was added to the receptor solution, and the mixture was incubated at room temperature for 30 min. The cross-linked receptor was subjected to SDS-PAGE analysis.

DD-polyornithyl gel electrophoresis (DEAE-DEAE): Various active fractions obtained during partitioning and gel filtration receptor preparations were analyzed by DEAE-DEAE as described by Lambe et al. (2). Samples were analyzed on a 15% SDS-PAGE gel, and the gel was stained with Coomassie blue.

Results

The FSH receptor of bovine calf testis was purified by differential centrifugation, followed by chromatography on DEAE-cellulose and hydroxyapatitecolumns. The purified receptor was then subjected to cross-linking experiments and analyzed by SDS-PAGE.

Cross-linking experiments revealed that the FSH receptor exists as a homodimer in the testis. The receptor was also found to be associated with other proteins, including a 60-kDa protein that may be involved in receptor trafficking.

Conclusion

The FSH receptor of bovine calf testis is a heterotetrameric protein that exists as a homodimer. Further studies are needed to understand the role of the associated proteins in receptor function and regulation.
FSH Receptor of Bovine Calf Testis

Molecular assembly and allogenic nature of the follitropin receptor—
mixed hamster testis (490-340) was radiolabeled as described to determine the kinase activity of the receptor. The kinase activity was determined by measuring the incorporation of 

4) Two sample aliquots were incubated in 6 x 6 ml area (final), for 6 h at 31°C. The other sample was then analyzed by autoradiography.
5) A third sample aliquot was incubated in 26 SDS (final) for 90 s at 100°C and analyzed by SDS-PAGE.
6) Other sample aliquots were incubated in 26 SDS (final) and 1, 2, 15, 70, or 200 mM DT (final) for 90 s at 100°C and analyzed by SDS-PAGE.
7) Other sample aliquots were incubated in 26 SDS (final) alone on 26 SDS and 10 mM DT (final) for 90 s at 100°C. The treated samples were analyzed by SDS-PAGE followed by autoradiography.

Enzymatic digestion of receptor/substrates—Purified receptor and substrate were analyzed by proteolytic digestion according to the procedure of Cleveland et al. (27). Radiolabeled follitropin receptor preparation was first resolved in SDS gels (15 μl acrylamide) under reducing and reducing conditions, as described above. The components of interest (containing 250 K and 60 K bands) were sliced, dissolved in the sample wells of a second SDS gel (15 μl acrylamide) and each gel overlayed with a final concentration of 25 uM of tropomyosin or testis proteins. Digestion proceeded directly in the streaking gel during subsequent electrophoresis. After electrophoresis, the gels were dried prior to autoradiography at -80°C.

RESULTS
Preparation and concentration of the 496-340 g receptor—In each calf testis 250 K and 60 K bands were decomplexed and an average wet tissue weight of 6,000 g was homogenized as described in MATERIALS TO obtain a total tissue homogenate of 25 liters containing an average protein yield of 493 g of protein/25 ml. Follitropin receptor concentration, low affinity and high capacity FSH-binding sites were removed to 7,000 g sediment. The detailed characteristics of these fractions have been previously reported (31). A 20-fold concentration of 7,000 g supernatant by ultrafiltration using Amicon DC-10 unit and a hollow fiber cartridge (101-50) with an exclusion limit of 50,000 Da, resulted in a protein recovery of 93% with a specific activity of 116 fmol/mg protein.

Purification of testicular light plasma membranes—A total of 1.1 liter of concentrated 7,000 g supernatant, obtained from a 11.5% by weight of calf testes was processed in the sucrose density gradient (10-30%) centrifugation, using a large-capacity zonal rotor (70-10). Significant amounts of 75 K and 50 K of liquid and inactive protein were separated in 260 fraction 1 between sucrose concentrations of 6% and 17.5%. 260 fraction II eluted between sucrose concentrations of 22.5% and 24% and contained high specific 125I-FSH-binding activity, and low 225I-FSH-binding activity. Measurements of 125I-concentration activity, supergrip and binding and hormone-induced edegate cyclase activity in individual fractions were previously reported (31). Several preparations of 260 fraction 10 (depicted) displayed lower concentrations of high affinity receptors for FSH (Table I) with average specific activity of 70 fmol/mg protein. Approximately 3.2 liters of 260 fraction 11, obtained from 11.5 liters of 260 fraction II, was eluted at 24% by sucrose gradient centrifugation, collected directly, dialyzed against washing buffer and concentrated by Amicon DC-10 unit using 50 ml at 50 KHollow fiber chromatography (101-50) with 70 K NaCl) without loss of hormone-binding capacity (Table I).

Fig. 1: Preparative centrifugation in iodine sucrose density of concentrated 7,000 g supernatant. Approximately 12.9 g of protein was loaded over a linear sucrose density gradient (50 ml), in a 10 ml rotor (70-10) at 1.6 liter capacity with 15% by weight of a Beckmann ultracentrifuge (Model 1L11). The rotor was accelerated to 2,600 rpm and the sample was then eluted at the same sucrose density gradient centrifugation was performed for 4 h at 4°C in a Beckmann ultracentrifuge (Model 1L11). The rotor was centrifuged at 15,000 rpm and the contents were eluted in 50 ml portions by displacement with 40% sucrose solution. Fractoins were pooled into three major fractions (260 fractions I, II, and III) as indicated by arrows in the figure based on protein content and receptor activity profiles of individual fractions.

Oligomerization of the follitropin receptor in Triton X-100: The follitropin receptor was solubilized from DC-2 concentrate at a protein concentration of 20 μM of 0.2% Triton X-100, representing a optimum detergent to protein ratio of 0.8:1. A simultaneous treatment of the Triton X-100-solubilized fraction with DCC-2 concentrate cation exchange absorbed iodinated ligands in the organic phase and prevented in the salting-out activity compared to untreated membranes. The aqueous phase was further centrifuged at 45,000 g for 1 h. The solubilized receptor fraction (supernatant) retained 75% of protein with a full retention of specific 125I-FSH-binding activity. The HCG receptor activity of this fraction, however, was much lower (47%) than that of the similarly treated sample prior to concentration. After several washes, an average recovery of detergent-soluble fraction was 2.4 g of protein, and its specific FSH-binding activity was 116 fmol/mg protein.

A flow chart of the procedure developed for further purification of follitropin receptor from detergent-soluble fraction of DC-2 concentrate, is given in Figure 2.

![Fig. 2: A flow chart of a typical procedure developed for the purification of follitropin receptor from the detergent-soluble fraction of calf testis lightest membranes.](http://www.jbc.org/)

Downloaded from http://www.jbc.org/ on October 20, 2017.
DEAE-cellulose chromatography: Approximately 32.7 mg protein aliquots of Sepharose 4B-Fr-2 were further purified each time by ion exchange chromatography. As shown in Figure 4, most MGO-binding, OppMNG-binding activities, and residual 2'-nucleotidease enzyme activity present in 4B-Fr-2 were absorbed by DEAE-cellulose and could be eluted by 0.3 M ammonium acetate, pH 5.8 ([Fr-3]) and 0.2 M ammonium acetate, pH 4.3 ([Fr-4]). The FSH-binding activity of 4B-Fr-2, however, was adsorbed by DEAE-cellulose and could be reabsorbed in breakthrough ([Fr-1]) and buffer wash ([Fr-2]) fractions. The average recovery of protein in these fractions was 95% of protein. Fractons 180-Fr-1 and DEAE-Fr-2 contained high affinity follitropin receptors (Table 1) with specific activity 36-fold higher than in 4B-Fr-2. The OppMNG-binding activity in DE-Fr-1 and DE-Fr-2 was extremely low, and an excellent separation between the two activities was achieved by DEAE-cellulose chromatography (Fig. 6).

LESTIC affinity chromatography: Fractons DE-Fr-1 and DE-Fr-2 were pooled and further purified by affinity chromatography using agarose-linked sheep serum follitropin (GnRH). A kinetic assay specifically binds to K-ethyl glucosamine and sialic acid residues. As shown in Figure 5, greater than 80% of the total FSH receptor activity present in fractions (DE-Br-1 and DE-Fr-2) was adsorbed by immobilized lectin, and could be eluted with 0.3 M K-ethyl glucosamine ([Ma-Fr-1]) and 2.4 M K-ethyl glucosamine ([Ma-Fr-4]). Seven amounts of OppMNG-binding activity present in the applied sample were unabsorbed by the lectin. The rotating fractions ([Ma-Fr-3B] and [Ma-Fr-4]) contained purified high affinity follitropin receptors (Table 2). Each average FSH-binding capacity of 3.47 (1.4-4.3) nM/ml protein which corresponds to 0.02% of FSH bound per ml 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 ovine of final extract). A 4% recovery of protein (i.e., 1.050 x g supernatant) represents an yield of only the high affinity and low capacity binding sites present in this fraction. Therefore, in Table 1, the recovery of total receptor activity and yield at different stages of purification are expressed as percent of total receptor activity initially present in this fraction. Approximately 52% of high affinity FSH receptor protein was recovered after lectin affinity chromatography. From 11.5 kg of ovine final extract, this represents a 73.3% yield of 0.008 x g supernatant. The purified receptor, when stored in 20 mM Hepes buffer, pH 7.5, containing 1.0 M NaF, 0.016% NaN3, 0.02% Triton X-100 and 0.2 M glycerol, was stable for several months at 4°C, or for a week at -80°C.
Characterization of the Follitropin Receptor

SDS-polyacrylamide gel electrophoresis. The purified fractions obtained after lectin affinity chromatography, when treated with 2% SDS alone for 90 min at 100°C, gave a single band with $K_r = 240,000$ as compared to 29-30 bands shown by the lighter membrane preparation fraction, indicating a high degree of purity and electrophoretic homogeneity of the purified receptor (Fig. 6). After treatment with 2% SDS-dithiothreitol (SDT) or 2% SDS for 90 min at 100°C, the purified receptor showed a single minor stained band with $K_r = 66,000$ (Fig. 6).

Heterodimerizing capacity lectin analysis of the 15% SDS-polyacrylamide-binding site in all active fractions obtained during various steps of purification (Table 2). The dissociation constant (K) of receptor potent in degenerate (K) construct testicular fraction,polymeric 680 fraction (f) and PAC fraction 10 were 0.46 x 10^{-6} M, 1.8 x 10^{-7} M, and 3.9 x 10^{-10} 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 lightly purified follitropin receptor ranged from 1.7 - 4.1 nmol of SDS/mg of protein.

Specificity of 125I-FSH-binding to the purified receptor preparation - A major criterion 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 125I-FSH to purified receptor in a concentration-related manner.  FSH, hCG and hCG did not inhibit 125I-hCG-binding at concentrations up to 50 ng/ml (Km [hCG]) or 25 ng/ml (hCG). These results substantiate a high degree of hormone specificity as well as absence of species specificity in FSH interaction with the purified receptor.

![Figure 3: Scatchard plot for the binding of 125I-FSH to purified FSH receptor.](http://www.jbc.org/)

**Figure 3**: Scatchard plot for the binding of 125I-FSH to purified FSH receptor. The purified receptor was incubated with a constant amount of 125I-FSH and increasing amounts of unlabeled FSH as described under METHODS. The data were expressed in the form of Scatchard plot.

![Figure 4: Specificity of 125I-FSH-binding to the purified receptor preparation.](http://www.jbc.org/)

**Figure 4**: Specificity of 125I-FSH-binding to the purified receptor preparation. Aliquots (7.5 ng of protein each of purified receptor after lectin affinity chromatography) were incubated with 125I-FSH (5 ng for 18 h at 4°C), together with indicated concentrations of unlabeled FSH (○), hCG (■), hCG (□), hCG, hCG (○) as indicated in the absence of unlabeled FSH as described under METHODS. Results (R/B) are expressed as the percent of specific 125I-FSH binding as described in the absence of unlabeled FSH as determined by assays. Each data point represents the mean of triplicate measurements.
The oligomeric nature of the FSH receptor-lectin affinity purified P2S receptor preparation was radiolabeled to specific activities of 15–62
KIU/mg. Following treatment of the receptor with 35 mM for 90 s at 100°C, and
analysis by SDS-PAGE and autoradiography, a major band with an apparent Mr of
340,000 was observed (Fig. 9A). Occasionally, an additional fast band
 correponding to Mr of 170,000 was also noticed (Fig. 9A). This component,
however, was unobserved by an 480-Kd lectin IF affinity column, whereas the
receptor was adsorbed and could be eluted by 50 ml sodium acetate buffer, pH
5.5, containing 11 M I (data not shown). Treatment of radiolabeled receptor with
4 or 5 s urea for 4 h at 5°C, prior to SDS-PAGE, did not alter the molecular
weight (Mr ~ 240,000) of the receptor (Fig. 13) suggesting that the receptor does
not contain non-covalently associated subunits. However, the treatment
with 2% SDS for 90 s at 100°C, followed by reducing final SDS concentration
to 0.1% by dialysis and the treatment with DTT (1 to 70 mM) for 45 s (at
5°C) induced a band of Mr ~ 240,000 band with appearance of an Mr ~ 60,000
band (Fig. 9A) Exposure of the receptor preparation to 200 mM of DTT did
not further dissociate the Mr ~ 60,000 band. Moreover, the band with Mr of
60,000 identified in 7 M acrylamide gel under reduced conditions, gave a
single band following electrophoresis in the second dimension in 13%
acrylamide gels (Fig. 11). Also, the purified receptor was incubated with
[125I]I-22K in the presence and absence of unlabeled P2S and the hormone-
receptor complexes were cavellently cross-linked with SDS. Analysis of cross-
linked complexes by SDS-PAGE under non-reducing conditions followed by
autoradiography revealed a single prominent band in the region of Mr ~ 300,000
(Fig. 12). To further understand the relationship between 240,000 and 60,000
bands of receptor, we have subjected both radioactive bands to peptide
mapping by limited proteolysis by elastase/tissue plasminogen activator
(TEPA) protease. SDS-PAGE analysis (Fig. 13) revealed that the pattern of the peptides produced after
partial proteolytic cleavage, were identical for both 240 kDa and 60 kDa
bands.
Fig. 12: Covalent cross-linking of 125I-FSH to purified FSH receptor. Unlabeled receptor after SDS-polyacrylamide gel filtration, and cross-linked by the bifunctional reagent disuccinimidyl suberate, as described in METHOD. The cross-linked receptor-hormone complexes were analyzed by SDS-PAGE under non-reduced conditions in 8% acrylamide separation gels containing 3.1% SDS, followed by autoradiography. The positions of markers 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 kDa and 60 kDa bands) were sliced, inserted in the sample wells (lane 1 and lane 2, respectively) of a second SDS gel (15% acrylamide) and overlayed with gel with Staphylococcus aureus protease (final concentration of 60 μg/ml). Electrophoresis was performed in the usual manner with the exception that the current was turned off for 30 min when the bromphenol blue dye in the control well neared the bottom of the stacking gel. Digestion proceeded directly in the stacking gel. An autoradiogram of a dried gel is shown.
Purification of follitropin receptor from bovine calf testes.
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