Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid

(ovary/pituitary/follicle-stimulating hormone/luteinizing hormone/microsequencing/gonadotropins)

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ABSTRACT A M_r 32,000 protein with inhibin activity was isolated from porcine follicular fluid by heparin-Sepharose affinity chromatography, gel filtration on Sephacryl S-200, and four reversed-phase HPLC steps. The isolated molecule is composed of two chains having molecular weights of 18,000 and 14,000, respectively, and bound together by disulfide bonds. Amino acid sequence analysis revealed the 10 NH_2 -terminal residues of the M_r 18,000 chain to be Ser-Thr-Ala-Pro-Leu-Pro-Trp-Pro-Trp-Ser- and those of the M_r 14,000 chain to be Gly-Leu-Glu-Xaa-Asp-Gly-Arg-Thr-Asn-Leu-. This M_r 32,000 protein specifically inhibits the basal secretion of FSH, but not that of LH, in the rat anterior pituitary monolayer culture system, with a half-maximal effective dose of 450 pg/ml.

The concept of inhibin as a water-soluble substance produced in the male gonad but acting specifically at the pituitary level to inhibit the secretion of gonadotropins was postulated by McCullagh in 1932 (1). This concept was later refined to preferential regulation of the secretion of follicle-stimulating hormone (FSH), prompting many laboratories in the past 50 years to isolate and characterize this substance from extracts of testis, rete testis fluid, seminal plasma, and ovarian follicular fluid (reviewed in refs. 2 and 3), using various bioassays. Although many reports have appeared claiming the purification of inhibin-like material with molecular weights ranging from 5000 to 100,000, subsequent studies have shown that these substances either were not homogeneous or did not have the high specific activity expected of true inhibin (reviewed in refs. 2 and 3). The difficulties encountered in the isolation of inhibin are best explained by the lack of a reliable bioassay that can specifically measure the substance and by the apparent self-aggregation or attachment of inhibin to a large carrier protein to yield multiple molecular species. These two problems were overcome by the introduction of the rat anterior pituitary monolayer culture system, which specifically and reliably detects the inhibition of basal FSH secretion exerted by inhibin (4, 5), and the use of denaturing solvents in the purification. We report the isolation, subunit composition, and NH_2 -terminal amino acid sequences of a M_r 32,000 protein with inhibin activity from porcine follicular fluid (PFF).

MATERIALS AND METHODS

Inhibin Bioassay. Throughout the purification procedure, inhibin bioactivity was monitored by an *in vitro* bioassay using rat anterior pituitary monolayer culture (6). In brief, anterior pituitaries of 21-day-old female rats were collected, enzymatically dispersed, and plated in 10% fetal bovine serum in Hepes-buffered Dulbecco's modified Eagle's medium (HDMEM) (GIBCO) into 24-well tissue culture plates

(Falcon) on day 1. On day 2 the medium was changed to 1% fetal bovine serum in HDMEM plus sample. Incubation was continued for another 48 hr. The medium then was harvested, and the luteinizing hormone (LH) and FSH present were determined by RIA, using materials provided by the Pituitary Hormone Program of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. In this assay the inhibin substance inhibits the basal release of FSH but not that of LH, as compared to release by control cells that receive the incubation medium only. For the detection of inhibin activity in the various column fractions, aliquots ranging from 0.01 to 0.1% by volume were removed, mixed with 100 μg human serum albumin in 100 μl of water, and dried in a Speed-Vac concentrator (Savant). The residue was dissolved in 3 ml of 1% fetal bovine serum in HDMEM, filtered through a Millex-GS 0.22- μm filter (Millipore), and assayed in duplicate.

Heparin-Sepharose Affinity Chromatography. A bottle containing 500 ml of frozen PFF (collected by J. R. Scientific, Woodland, CA, and procured through the Contraceptive Development Branch, Center for Population Research, National Institute of Child Health and Human Development) was defrosted, and the cell debris was spun down in a Beckman J2-21 centrifuge in a JA-20 rotor at 10,000 rpm for 30 min. One-half of the supernatant (250 ml) was diluted to 10 times its volume (2500 ml) with 0.1 M NaCl/10 mM Tris Cl, pH 7, in a 4-liter Erlenmeyer flask and pumped simultaneously via eight Silastic tubings (0.76 mm i.d.) into eight heparin-Sepharose (Pharmacia) columns (3.5 $\times$ 9 cm) by two Rabbit four-channel peristaltic pumps (Rainin Instruments, Emeryville, CA) at 40 ml/hr per column. After all the fluid had been pumped through the heparin-Sepharose, the eight columns were washed simultaneously with 3.5 liters of 0.1 M NaCl/10 mM Tris Cl, pH 7, in the same manner. The adsorbed proteins were removed by washing the eight columns simultaneously, as above, with 1.3 liters of 1 M NaCl/10 mM Tris Cl, pH 7. Fractions (16 ml) were collected, and inhibin activity was monitored by the *in vitro* bioassay. The columns were regenerated by further washing with 1.6 liters of 2 M NaCl/10 mM Tris Cl, pH 7, and reequilibrated with 3.5 liters of 0.1 M NaCl/10 mM Tris Cl, pH 7, for purification of the remaining 250 ml of PFF.

Sephacryl S-200 Superfine Gel Filtration. Fractions containing inhibin activity eluted from the eight heparin-Sepharose columns were pooled (400 ml) and dialyzed overnight (Spectra/Por 3 membrane tubing, 28.6 mm cylindrical diameter, M_r cutoff 3500; Spectrum Medical Industries, Los Angeles) against 16 liters of 30% (vol/vol) acetic acid. The retentate was centrifuged (JA-20 rotor, 10,000 rpm, 30 min) to remove a white precipitate, and the supernatant divided

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Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; PFF, porcine follicular fluid.

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into eight equal portions for application to eight 5×100 cm columns of Sephacryl S-200 Superfine (Pharmacia). Each column was developed with 30% acetic acid at 20 ml/22 min and the column fractions were monitored by absorption at 280 nm and bioassay.

Reversed-Phase HPLC. The fractions containing inhibin activity recovered from the eight Sephacryl S-200 columns were pooled and lyophilized. The lyophilized material (40 mg) was dissolved in 40 ml of 0.2 M acetic acid and filtered through a Millex-HA 0.45- μ m filter (Millipore). The filtrate was applied directly to a HPLC column. After all the filtrate had been loaded, the column was washed with the aqueous buffer A until the UV absorption reached baseline. The inhibin activity was separated in a Beckman 332 gradient liquid chromatography system equipped with a Spectroflow 757 UV detector (Kratos Analytical Instruments, Ramsey, NJ), a Soltec 220 recorder (Soltec, Sun Valley, CA), and a Redirac 2112 fraction collector (LKB). Two reversed-phase HPLC solvent systems were used. In the Et_3NP system, solvent A consists of 0.25 M triethylammonium phosphate (pH 3), and solvent B is 80% (vol/vol) acetonitrile in buffer A. In the CF_3COOH system, solvent A contains 1 ml of trifluoroacetic acid in 999 ml of water, and solvent B is 1 ml of trifluoroacetic acid in 199 ml of water and 800 ml of acetonitrile. Other reversed-phase HPLC conditions are given in the legends to Figs. 1 and 2.

NaDodSO₄/PAGE. The inhibin proteins isolated from the final reversed-phase HPLC purification was analyzed under reducing and nonreducing conditions in 1-mm-thick 15% acrylamide gel according to the method of Laemmli (7). The proteins were revealed by silver-staining reagent (Bio-Rad). The following molecular weight standards were used to calibrate the gel: bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), α -chymotrypsinogen (M_r 25,700), and lysozyme (M_r 14,500). For nonreducing conditions, 2 μ g of inhibin protein in 20 μ l of water was incubated with 20 μ l of sample buffer (0.152 M Tris Cl, pH 6.8/20% (vol/vol) glycerol/4% NaDodSO₄/0.04% bromphenol blue) for 1 hr at 37°C and then loaded onto the gel. Electrophoresis was at constant voltage (200 V) for 6 hr at room temperature. For reducing conditions, 2 μ g of protein was incubated first with 20 μ l of 0.02 M dithiothreitol for 15 min at 37°C, and then 20 μ l of sample buffer was added and the incubation was continued for 1 hr before the sample was applied to the gel. Electrophoresis was carried out as above, except that 5 mM dithiothreitol was included in the electrophoretic buffer.

Amino Acid Analysis and Microsequencing. Amino acid analyses and NH_2 -terminal sequence determination of the intact inhibin proteins were performed as described (8, 9). NH_2 -terminal sequence analyses of the M_r 18,000 and 14,000 chains of inhibin protein B were accomplished by first separating the two chains by NaDodSO₄/PAGE under reducing conditions, followed by electroblotting of the separated protein onto GF/C paper which was subsequently punched out for direct microsequencing. Briefly, inhibin protein B (12–15 μ g) was electrophoresed in 0.5-mm-thick 15% acrylamide gel (8×10 cm) under reducing condition as above. After electrophoresis, the gel was immediately washed three times with 0.5% Nonidet P-40 (Sigma) in 0.5% acetic acid at room temperature. The gel was then placed between two sheets of trifluoroacetic acid-activated GF/C paper (Whatman) that had been wetted briefly with the blotting buffer (2% acetic acid containing 0.5% Nonidet P-40). One more activated GF/C paper was placed on the cathodic side in case the protein was not completely absorbed by the first paper. The outer surface of the GF/C paper was protected by a Whatman no. 3 filter paper, and the whole assembly was inserted between two Scotch-Brite pads held together by a plastic grid (Bio-Rad). Electrophoresis in the blotting buffer, at constant voltage (60 V) for 16 hr at 4°C, was

used to transfer the positively charged proteins onto the GF/C paper. The electrophoretically transferred proteins were revealed by Coomassie blue staining. Circles (1.2 cm diameter) were punched out of the stained protein bands and subjected directly to microsequencing as described (9).

RESULTS

Approximately 18 liters of frozen PFF was processed in batches of 250 ml to isolate the inhibin proteins. The first step of the purification was heparin-Sepharose affinity chromatography, and the adsorbed inhibin proteins were recovered with 1 M NaCl elution. This step greatly expedites the purification procedure because a relatively large volume of PFF can be processed and the recovery of the total inhibin activity is at least 90%. To save time during the purification process, only fractions in the eluent region expected to contain inhibin activity were assayed for inhibition of basal FSH secretion. The active substance from the 1 M NaCl eluate of the heparin-Sepharose columns was pooled and, after dialysis to remove most of the NaCl, further purified by Sephacryl S-200 gel filtration using a denaturing solvent (30% acetic acid) as eluent (Fig. 1a). Two zones of approximately equal activity were detected, one of apparent $M_r \approx 30,000$ and the other of apparent $M_r \approx 12,000$ (see below for further measurements of M_r). However, based on the protein concentration as determined by UV absorption, the low-molec-

FIG. 1. (a) Sephacryl S-200 gel filtration of the PFF inhibin proteins. The effluents from the 1 M NaCl wash of the eight heparin-Sepharose columns were pooled, dialyzed, and divided into eight equal portions for gel filtration. The profile from one of the Sephacryl columns is shown. (b) Reversed-phase HPLC purification of the inhibin proteins recovered from gel filtration. The active-region fractions (solid bar in a) were pooled, lyophilized, dissolved in 0.2 M acetic acid, and then applied to a Vydac C₄ column and eluted with the indicated gradient of acetonitrile in Et_3NP buffer at 9 ml/3 min. Two zones of inhibin activity (A and B, indicated by the solid bars) were recovered.

ular-weight zone contains much higher specific activity. As a result, this region was selected for further purification. The low-molecular-weight inhibin protein from the eight S-200 columns was pooled and lyophilized. The lyophilized material was loaded directly onto a 1×25 cm Vydac 5- μ m-particle-size C_4 column (The Separations Group, Hesperia, CA) and developed with an acetonitrile gradient in Et_3NP buffer (Fig. 1*b*). Again, two zones of inhibin activity were detected, with more activity exhibited by the earlier-eluted zone (A) than the later-eluted zone (B). Both zones A and B were separately pooled and their respective inhibin activities were further purified by two more reversed-phase HPLC steps, first using a 1×25 cm Vydac (5- μ m) C_4 column and

the CF_3COOH solvent system (Fig. 2*a* and *b*) and then using a 1×25 cm Vydac (5- μ m) phenyl column and the Et_3NP buffer system (Fig. 2*c* and *d*). Finally, inhibin proteins A and B accumulated from 4 to 10 batches were concentrated by reversed-phase HPLC using a 0.46×25 cm Aquapore RP-300 (10- μ m) column (Brownlee Labs., Santa Clara, CA) and the CF_3COOH solvent system (Fig. 2*e* and *f*). Altogether, ≈ 600 μ g and ≈ 60 μ g of inhibin proteins A and B, respectively, were purified from the 18 liters of PFF.

Amino acid analysis of inhibin proteins A and B shows that they are closely related but not identical (Table 1). Notable differences were found in the quantities of isoleucine, histidine and lysine. On NaDodSO₄/PAGE under nonreducing

FIG. 2. Reversed-phase HPLC purification of inhibin-like proteins A (Left) and B (Right). (a and b) Active fractions in zones A and B (solid bars in Fig. 1*b*), respectively, were pooled and, after being diluted to 3 times their original volume with 0.2 M acetic acid, applied directly onto a Vydac C_4 column and eluted with the indicated gradient of acetonitrile in the CF_3COOH system at 9 ml/3 min. (c and d) The active material (denoted by the solid bars in a and b, respectively) was pooled, diluted to 3 times its original volume, and chromatographed on a Vydac phenyl column with the indicated gradient of acetonitrile in the Et_3NP buffer system at 2 ml/2 min. (e and f) The active material accumulated from 4–10 chromatographic runs identical to those in c and d, respectively, was pooled and concentrated by chromatography on an Aquapore RP-300 column, using the indicated gradient of acetonitrile in the CF_3COOH system at 0.5 ml/min.

Table 1. Amino acid composition of purified inhibin proteins A and B from PFF

Amino acid	Residues per 32 kDa	
	Protein A	Protein B
Asx	18.5 ± 1.6	21.0 ± 1.1
Thr	11.5 ± 0.5	13.5 ± 0.6
Ser	16.3 ± 1.4	18.1 ± 0.7
Glx	21.3 ± 1.4	22.5 ± 0.5
Gly	19.9 ± 1.9	22.4 ± 0.7
Ala	19.7 ± 1.4	22.0 ± 0.6
Val	14.1 ± 1.8	14.4 ± 0.4
Met	6.1 ± 0.4	5.8 ± 0.3
Ile	14.6 ± 1.7	10.3 ± 0.2
Leu	27.3 ± 2.1	27.8 ± 0.8
Tyr	10.7 ± 1.3	11.4 ± 0.4
Phe	11.6 ± 1.4	10.0 ± 0.2
His	14.1 ± 1.2	7.9 ± 0.6
Trp	4.4 ± 0.3	3.9 ± 0.4
Lys	11.9 ± 0.9	5.4 ± 0.2
Arg	13.9 ± 0.6	15.8 ± 0.4
Cys*	14.4 ± 0.2	14.2 ± 0.9
Pro	29.2 ± 0.9	29.6 ± 1.1

Values represent mean ± SD of four analyses, normalized to a protein of 32,000 daltons.

*Determined as cysteic acid after performic acid oxidation.

conditions, inhibin protein B showed a single band migrating at M_r 32,000, whereas protein A showed at least three bands migrating closely together around M_r 32,000 (Fig. 3a). Under reducing conditions, inhibin protein B separated into two bands, one migrating at M_r 18,000 and the other at M_r 14,000, while protein A showed three groups of closely migrating bands at M_r 18,000, 14,000, and 8,000, respectively (Fig. 3b). Potency analysis showed that the purified inhibin proteins A and B exhibit half-maximal inhibition of basal FSH secretion at concentrations of 900 pg/ml and 450 pg/ml, respectively (Fig. 4). The lower potency of protein A is in agreement with its being less pure than protein B. As expected, both inhibin proteins inhibit the basal secretion of FSH but not that of LH.

Microsequencing from the NH_2 terminus of intact inhibin protein B consistently revealed two residues in approximately equal amounts at each cycle, indicating the protein is composed of two chains. The same two residues as in protein B were detected in approximately equal amounts in the first six cycles of Edman degradation when intact inhibin protein A was subjected to NH_2 -terminal sequence analysis. The two chains in protein B were separated by NaDodSO₄/PAGE

FIG. 3. NaDodSO₄/PAGE analysis of the purified inhibin proteins A and B under nonreducing conditions (a) and under reducing conditions (b). Positions of the molecular weight standards are indicated at left. Numbers represent $M_r \times 10^{-3}$.

FIG. 4. Dose-response curves for the purified inhibin proteins A (□) and B (○) and the crude PFF standard (Δ) on the basal secretion of FSH and LH from cultured rat anterior pituitary cells. The crude PFF standard was a "charcoal-stripped" and 40%-saturated ammonium sulfate precipitate of PFF (10). The half-maximal effective dose was calculated by the computer program BIOPROG (11).

under reducing conditions, and the separated chains were electroblotted onto GF/C paper. After the protein bands were detected with Coomassie blue, they were excised and subjected directly to microsequencing. Based on the results from multiple sequence analyses of both intact and reduced inhibin protein B, the first 10 NH_2 -terminal residues of the M_r 18,000 chain are Ser-Thr-Ala-Pro-Leu-Pro-Trp-Trp-Ser- and those of the M_r 14,000 chain are Gly-Leu-Glu-Xaa-Asp-Gly-Arg-Thr-Asn-Leu-. The unidentified residue of the M_r 14,000 chain is probably cysteine, since 14 cysteines were found by amino acid analyses of the intact molecule (Table 1) and no phenylthiohydantoin-amino acid residues could be identified in this cycle.

DISCUSSION

Two proteins (A and B) with inhibin activity were purified from PFF by sequential heparin-Sepharose affinity chromatography, gel filtration on Sephacryl S-200 developed in 30% acetic acid, and four reversed-phase HPLC steps. Protein B was found to be homogeneous with a molecular weight of 32,000 on NaDodSO₄/PAGE under nonreducing conditions. This apparent molecular weight is much higher than that determined by Sephacryl S-200 gel filtration. The lower molecular weight obtained for protein B by Sephacryl chromatography indicates that, in 30% acetic acid, protein B assumes a more compact conformation (smaller radius of gyration) than does the A protein. The intact protein B molecule is composed of two chains of M_r 18,000 and 14,000, respectively, and held together by disulfide bond(s). Apparently the inhibin activity requires both chains to be joined together, because reduction of the disulfide bonds by dithiothreitol completely destroyed the bioactivity. Inhibin protein A, which was isolated in much higher quantity than B, was found to be heterogeneous on NaDodSO₄/PAGE under both nonreducing and reducing conditions. Since the same two NH_2 -terminal sequences were found for each of the two proteins in the first six cycles of Edman degradation, A may be closely related to B in structure.

In the Sephacryl S-200 gel filtration an inhibin-like material of higher molecular weight than proteins A and B was detected. We do not know whether there is a structural relationship between this high-molecular-weight inhibin-like protein and the A and B species. The larger molecule could be a precursor or an aggregate of the M_r 32,000 inhibin protein. Indeed, Robertson *et al.* (12) have recently reported the isolation of a M_r 56,000 inhibin from bovine follicular

fluid. They also reported that the molecule was composed of two chains, of M_r 44,000 and 14,000, bound together by disulfide bonds. However, the first three NH_2 -terminal residues of the M_r 44,000 chain reported by Robertson *et al.* (12) are different from the first three residues of either of the two chains of the inhibin protein purified by us, although the second and third residues reported (12) for the M_r 14,000 chain are identical to those of the M_r 14,000 chain determined by us. We do not know what the relationship is between the M_r 32,000 inhibin protein we purified and the M_r 56,000 molecule isolated by Robertson *et al.* (12).

On the other hand, our inhibin protein is definitely different from the " α -inhibins" (13, 14) and " β -inhibin" (15) recently isolated and characterized from human seminal plasma. α -Inhibins are reported to be single-chain polypeptides all derived from a 92-residue parent molecule (14). However, neither a synthetic replicate of the 31 amino acid NH_2 -terminal fragment of " α -inhibin-52" nor the native product inhibited the basal FSH-releasing activity in the rat anterior pituitary cell culture (13, 16). " β -Inhibin" is described as a single-chain polypeptide with 94 amino acids, with the bioactive core residing at the COOH-terminal 67–94 fragment (17). We have synthesized this fragment but found it to be inactive, up to a concentration of 5 $\mu\text{g}/\text{ml}$, in inhibition of the basal secretion of FSH in the rat anterior pituitary cell culture assay (data not shown). It must be pointed out that the bioassay methods (for "inhibin activity") used in these two laboratories (13, 14) are substantially different from those used by us (6) in this study. Moreover, the recent report by Lilja and Jeppsson (18), who have characterized a molecule identical to α -inhibin-52 as "the main degradation product of the gel-forming protein secreted by the seminal vesicles," raises questions as to the biological significance of this material as reported (13, 14).

Finally, Matsuo and Igarashi and their co-workers have reported the isolation of a M_r 32,000 protein with inhibin activity from PFF, using a purification scheme different from ours but the same *in vitro* bioassay (19). The yield of their purified material was much higher than that of ours, possibly because they collected their own follicular fluid in the presence of enzyme inhibitors[†], whereas our starting material was collected under less than ideal conditions by commercial organizations. (Indeed, we have often experienced variations in the yield of the purified product between different bottles of the frozen fluid.) Matsuo and Igarashi and co-workers also reported that the M_r 32,000 inhibin was composed of a M_r 20,000 and a M_r 13,000 chain bound together by disulfide bond(s) (19, †). The sequence of the first four amino acids from the NH_2 terminus of the M_r 20,000 chain was Ser-Thr-Ala-Pro-, and that of the M_r 13,000 chain was Gly-Leu-Glu-Cys-. These two NH_2 -terminal sequences are identical,

respectively, to those of the M_r 18,000 and M_r 14,000 chains reported in the present paper. Thus, we tentatively conclude that the M_r 32,000 inhibin isolated in the laboratories of Matsuo and Igarashi and co-workers is probably identical to the M_r 32,000 inhibin protein isolated by us.

Note Added in Proof. We have now isolated the M_r 18,000 and 14,000 chains of inhibin protein A. Microsequencing has shown that the structure of the M_r 18,000 chain from inhibin protein A is identical to that of inhibin protein B, while the first 10 residues from the NH_2 -terminal end of the M_r 14,000 chain of inhibin protein A are Gly-Leu-Glu-Xaa-Asp-Gly-Lys-Val-Asn-Ile.

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