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Inhibin in immature rat Sertoli cell conditioned medium: a 32 kDa $\alpha\beta$-B dimer

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Summary

Conditioned medium of cultured Sertoli cells from 21-day-old rats was used as starting material for the isolation of inhibin. Inhibin activity was monitored by the dose dependent suppression of the follicle-stimulating hormone release of cultured rat pituitary cells. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of the highly purified inhibin preparation revealed a 32 kDa protein after silver staining, which could be separated in subunits of 18 kDa and 12 kDa after reduction. Western blot analysis with an antibody recognizing the 22 N-terminal amino acids of the α-subunit of 32 kDa bovine inhibin confirmed the presence of a 32 kDa inhibin molecule under non-reducing conditions, whereas an 18 kDa α-subunit was found after reduction. An antibody recognizing the β-A subunit of inhibin did not yield a signal after Western blotting. N-terminal amino acid sequence analysis of two highly purified preparations of inhibin obtained using different methods yielded the sequence predicted for a 32 kDa $\alpha\beta$-B dimer on basis of cDNA nucleotide sequence. This result is in agreement with the large excess of β-B over β-A mRNA in the rat testis.

Introduction

Inhibin is a gonadal glyprotein hormone consisting of two dissimilar disulphide-linked subunits, which inhibits pituitary gonadotrophin production and/or secretion, preferentially that of follicle stimulating hormone (FSH) (Burger and Igarashi, 1988). The isolation of inhibin has been hampered by the hydrophobic nature of the molecule, the use of different assay systems with the potential for detection of non-specific effects and the presence of different molecular weight forms of inhibin (de Jong, 1988). Inhibin is present in several gonadal fluids; the highest concentration of inhibin is found in ovarian follicular fluid (de Jong and Sharpe, 1976). Inhibin with a molecular mass of 32 kDa has been purified from both bovine (Fukuda et al., 1986; Robertson et al., 1986), ovine (Leversha et al., 1987) and porcine (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985) follicular fluid (bFF, oFF and pFF respectively). Upon reduction of 32 kDa inhibin two subunits (α- and β-subunit) of 18 and 14 kDa, respectively, were found. Ling et al. (1985) isolated two forms of inhibin from pFF. These

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forms were named inhibin A and B and differed in the N-terminal amino acid sequences of their β-subunits, which are now termed β-A and β-B. Subsequently, genes coding for the α, β-A and β-B-subunits have been cloned from human, porcine, bovine and rat cDNA libraries (for reviews see de Kretser and Robertson, 1989 and Vale et al., 1988).

Relatively little is known about the structure of testicular inhibin. Bardin et al. (1987) reported the purification of two forms of inhibin from ovine rete testis fluid, both composed of an αβ-A dimer but one with a truncated α-subunit. Partial characterization of inhibin from Sertoli cell conditioned medium has been reported for rat (Bicsak et al., 1987; de Jong et al., 1987) and cynomolgus monkey (Noguchi et al., 1987). Here, we report the isolation, subunit composition and N-terminal amino acid sequence of a 32 kDa inhibin isolated from rat Sertoli cell conditioned medium.

Materials and methods

Bioassay for inhibin

Inhibin-like bioactivity was determined using a bioassay detecting the suppression of spontaneous FSH release from incubated pituitary cells (Grootenhuis et al., 1989). Briefly, after a preincubation of pituitary cells for 3 days, media were changed and inhibin containing samples were added at two or three dose levels in triplicate. After a second 3-day culture period the concentration of FSH in the incubation medium was determined. A bFF preparation with an arbitrary potency of 1 U/μg protein was used as standard. The International Research Standard for Inhibin (86/890, Waites et al., 1987) has a relative specific activity of 60 ± 10 U/μg (mean ± SEM, $n = 5$) expressed in units of this bFF standard. Inhibin potencies were calculated using statistics for assay parallelism (Finney, 1964).

Sertoli cell culture

Sertoli cells were isolated from 21-day-old Wistar rats using a double collagenase digestion method as described by Oonk et al. (1985) and Grootenhuis et al. (1989). Briefly, approximately 3×10^6 Sertoli cells (8 mg protein) were plated in 150 cm^2 plastic flasks (Costar, Cambridge, MA, U.S.A.) in 1% fetal calf serum (FCS; Gibco, Grand Island, NY, U.S.A.) in minimal essential medium (MEM, Gibco). On the next day cells were washed and further cultured at 37°C in MEM, supplemented with antibiotics. Rat Sertoli cell conditioned medium (rSSCM) was collected every 3 or 4 days for 4–5 weeks and stored at −20°C.

Purification

Two pools of 5 l rSCCM were concentrated to a volume of 220 ml each under nitrogen pressure at 4°C using a filtration unit (Amicon, Lexington, MA, U.S.A.) using a hollowfibre (HIP10-43; Amicon) with a molecular weight cut-off at 10 kDa. The concentrate was repeatedly exchanged with buffer A of the Mono Q column (see below). Two different column sequences were used for the purification of inhibin, in both methods batches of 40 ml concentrated rSCCM were applied to a Mono Q anion-exchange column (HR 5/5) attached to a standard FPLC system with a GP-250 controller (Pharmacia, Uppsala, Sweden). After the OD_{280nm} profile reached baseline, retained proteins were eluted using a 30 ml linear gradient of 0–600 mmol/l NaCl in buffer A (Tris-HCl buffer; 20 mmol/l, pH 9.2) at 1 ml/min and 1 ml/fraction. The fractions containing the peak of bioactivity were pooled and purified according to one of the methods described below. Lentil Lectin-Sepharose (Pharmacia) chromatography of hollowfibre-concentrated rSCCM was performed according to de Jong et al. (1987).

Method 1. The pooled fractions, containing inhibin bioactivity from the Mono Q columns, were separated in five runs on a C1/C8 reversed phase column (HR 5/20, Pharmacia) using a 30 ml gradient of 0–50% (v/v) acetonitrile containing 0.1% (v/v) trifluoracetic acid (TFA) at 1 ml/min and 1 ml/fraction (de Jong et al., 1987) using an FPLC system. Bioactive fractions were lyophilized and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% gels (see below). After separation, 2 mm slices were cut and proteins were eluted and methanol precipitated as described by Grootenhuis et al. (1989).

Method 2. From a second batch of rSSCM the bioactive fractions from the Mono Q runs were also pooled. One-third of this pool peak of bioac-

tivity (an equivalent of 1200 ml rSCCM) was applied to a Bakerbond wide-pore C18 column (Baker Chemicals, Phillipsburg, NJ, U.S.A.) using a liquid chromatography system from Waters Associates (Milford, MA, U.S.A.), which consisted of a M-720 system controller, a M-6000A solvent delivery system, an M-441 UV/Vis absorbance detector, on-line equipped with a Shimadzu C-R3A chromatopac data module. The sample was fractionated using a 70 ml linear gradient of 20–48% acetonitrile in phosphate buffer (10 mmol/l; pH 6.8) at 1 ml/min, 1 ml/fraction and the protein profile was recorded at 214 nm. The bioactive fractions were pooled, dried by rotary evaporation in vacuo, applied on a C18 aquapore RP-300 column connected to a microbore 130A Separation System (Applied Biosystem, Foster City, CA, U.S.A.) and eluted with a 50 min linear gradient from 18–48% acetonitrile in 0.1% TFA at 200 μl/min and 400 μl/fraction. Proteins in the eluate were detected at 210 nm.

N-terminal amino acid sequence analysis

The amino acid sequence determination was performed on an Applied Biosystems model 470A protein sequencer, on-line equipped with a model 120A PTH analyzer.

SDS-PAGE

Proteins in the fractions of the last purification step were separated by SDS-PAGE (Laemmli, 1970) on gels of 15% (w/v) acrylamide and 0.8% (w/v) bisacrylamide, without or with prior reduction (5 min 100°C, 1% (v/v) β-mercaptoethanol).

Isoelectrofocussing

Isoelectrofocussing was performed on gels with a pH gradient of 3–9 using the Phast gel system (Pharmacia) according to manufacturer's application notes. For calibration an isoelectrofocussing kit (Pharmacia) was used.

Silver staining and Western blot analysis

For silver staining, gels were stained with Page Blue 83 (Bakker et al., 1987), incubated 1 h with 10% glutaraldehyde, washed overnight with distilled water and stained according to the method of Wray et al. (1981).

For Western blot analysis, separated proteins were electroblotted onto nitrocellulose and incubated with an antiserum against the 22 N-terminal amino acids of the α-subunit of bovine 32 kDa inhibin, which was described earlier by Grootenhuis et al. (1989).

Results

Sertoli cell conditioned medium

Immature rat Sertoli cells cultured in medium without serum at 37°C produce appreciable amounts of bioactive inhibin (Fig. 1). After an initial significant rise of the inhibin concentration in the medium in the second week, large amounts of bioactive inhibin are produced up to the fifth week. The relative specific activity (RSA) of the two pools of rat Sertoli cell conditioned (rSCCM) medium were 20.6 and 17.1 when compared with the bFF standard (see Table 1). Recovery of inhibin bioactivity after concentration of the pooled media was 121% and 95% for the first and second pool, respectively.

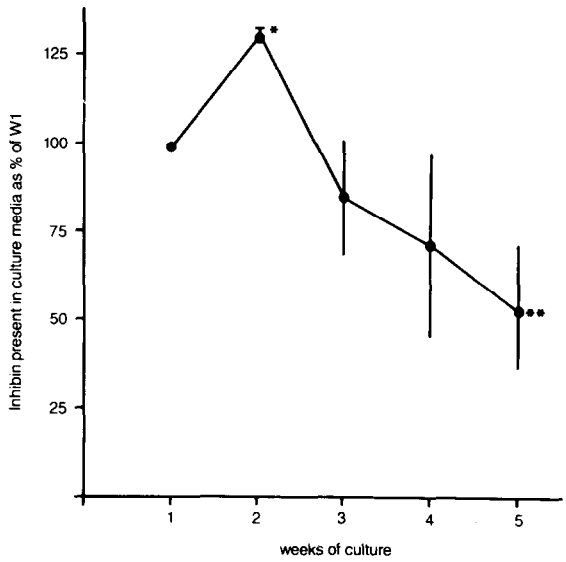

Fig. 1. Time-dependent production of bioactive inhibin by Sertoli cells, derived from 21-day-old rats cultured at 37°C. Media were changed twice a week for 5 weeks. Media from 1 week were pooled and assayed together. Values are obtained from three different Sertoli cell preparations and are expressed as percentage of the value for week 1 (means ± SEM; $*P < 0.05$ compared with week 1, $**P < 0.05$ compared with week 2 (paired Student's *t*-test)).

TABLE 1

RELATIVE SPECIFIC ACTIVITY (RSA) AND RECOVERY OF INHIBIN AFTER SEVERAL STEPS DURING ITS PURIFICATION FROM RAT SERTOLI CELL CONDITIONED MEDIUM (rSCCM)

The amount of rSCCM used in the purification with two different methods is indicated, and fractions (fr) used for the following purification steps are listed. Protein content was determined using: [a]Bradford, [b]OD_{280nm}, or [c]amino acid analysis.

Preparation	Total units ($\times 10^3$)	RSA (U/mg) ($\times 10^3$)	Recovery at each step (%)	Purification factor
Method 1				
rSCCM (4100 ml)	1,340	20.6 [a]	100	1
> 10 kDa	1,620	34.7 [a]	121	1.7
Mono Q fr 9+10	983	88.2 [b]	61	4.3
C1/C8 fr 5, 6, 7	189	121 [b]	28	5.9
SDS-PAGE 29–32 kDa proteins	123	16,400 [c]	65	800
Method 2				
rSCCM (1200 ml)	452	17.1 [a]	100	1
> 10 kDa	429	21.5 [a]	95	1.26
Mono Q fr 10, 11, 12	174	41.3 [b]	40	2.5
C18/pH 6.8 fr 29–40	43	114 [b]	17	6.7
C18 0.1% TFA fr 18	1,156	1,300 [c]	5.3	76

Purification of rat Sertoli cell inhibin

Batches of approximately 13 mg of Amicon concentrated rSCCM protein were chromatographed on Mono Q anion exchange columns. Inhibin bioactivity eluted between 140 and 260 mmol/l NaCl (fraction 8–13, Fig. 2). The recovery of applied inhibin bioactivity in the pooled bioactive fractions from the Mono Q columns was 61% and 40% for the first and the second pool of rSCCM, respectively. We also used Lentil Lectin-Sepharose chromatography of Amicon concentrated rSCCM. From this column $94 \pm 7.6\%$ (mean $\pm$ SEM, $n = 5$) of the recovered bioactivity could be eluted with 0.2 M α-methylmannoside, with no apparent increase in RSA.

For *method 1*, pooled bioactive fractions from the Mono Q columns with an RSA of 88.2 were separated in five runs on a C1/C8 column, using a gradient of acetonitrile containing 0.1% TFA. Inhibin bioactivity was found in fractions 5, 6 and 7 with an RSA of 425. However, after lyophilization RSA dropped to 121, indicating a 72% loss of bioactivity during this procedure. This material (1.56 mg) was separated by SDS-PAGE, eluted, methanol precipitated and analyzed for inhibin

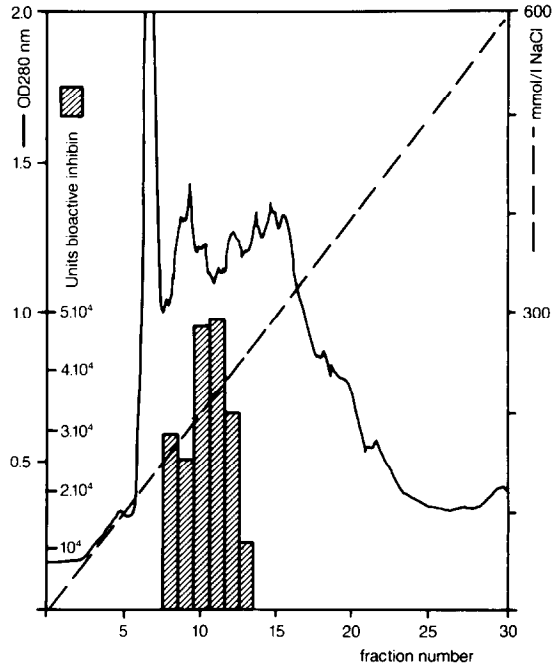

Fig. 2. Anion-exchange chromatography of concentrated rSCCM on a Mono Q column. Proteins (———, OD_{280nm}) were eluted with a lineair gradient of 0–600 mmol/l NaCl (— — —) in a 20 mmol/l Tris-HCl buffer pH 9.2. The hatched area represents inhibin bioactivity.

bioactivity. In eluates of slices 18 and 19, containing proteins of 29–33 kDa, 65% of the applied bioactivity was recovered. On basis of amino acid sequence analysis, 7.5 μg protein was present in these eluates, resulting in a 800-fold purified inhibin preparation with an RSA of 16.4×10^6.

For *method 2*, bioactive fractions from a second pool of rSCCM separated on Mono Q columns, were combined. Proteins of this pool were separated on a C18 column in a phosphate buffer at pH 6.8. Inhibin bioactivity was found in fractions 25–40, with a recovery of 25% of the applied bioactivity (Fig. 3). Fractions 29–40, containing the peak of inhibin activity, were pooled, partly lyophilized and subsequently loaded onto an RP-300 C18 column. Proteins were eluted with a gradient of acetonitrile containing 0.1% TFA. All eluted inhibin bioactivity was found in fraction 18 (Fig. 4) although most of the activity was lost (5% recovery). This eluate was used for amino acid sequencing.

One-third of the Mono Q pool was purified in parallel. Inhibin bioactivity was again found in fraction 18 from the C18 column developed with the TFA buffer. The RSA of this inhibin preparation was 1.3×10^3. This indicates a 76-fold purification factor compared with rSCCM, with a yield of 0.035%. This preparation was also used for amino acid sequencing.

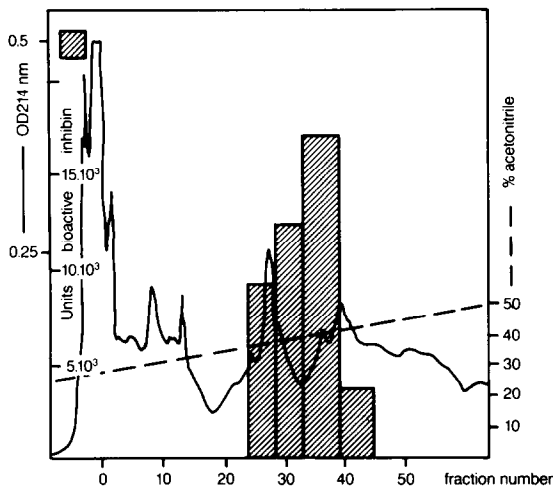

Fig. 3. Reversed-phase chromatography of the inhibin containing fractions from the Mono Q column (Fig. 2) on a C18 column. Proteins (———, OD_{214nm}) were eluted with a lineair gradient of acetonitrile (— — —) in a phosphate buffer (10 mmol/l; pH 6.8). The hatched area represents inhibin bioactivity.

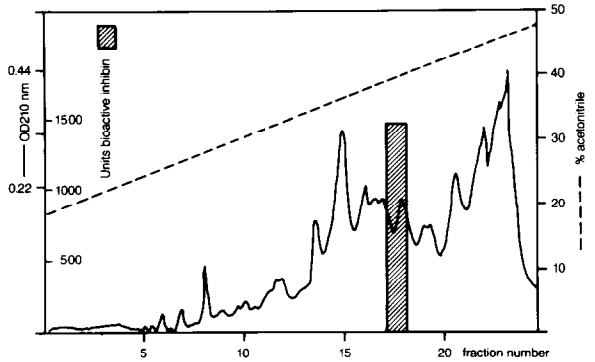

Fig. 4. Reversed-phase chromatography of the inhibin containing fractions (29–40) from the C18 column in Fig. 3 on a C18 column developed with a gradient of acetonitrile (— — —) in a buffer containing 0.1% TFA. Proteins (———, OD_{210nm}) and inhibin bioactivity (hatched area) are indicated.

Analysis of purified inhibin

SDS-PAGE. Fractions of the latter column were analyzed by SDS-PAGE. Part of the gel was silver stained, whereas another part was used for Western blot analysis using an antibody against the 22 N-terminal amino acids of the α-subunit of 32 kDa bovine inhibin. The appearance of immunoreactive inhibin α-subunit (Fig. 5) coincided with the presence of bioactive inhibin in fraction 18. Under non-reducing conditions a 32.5 kDa immunoreactive band was found. After reduction the apparent size of the immunoreactive protein was 19 kDa. The same bioactive fraction was also analyzed (with or without prior reduction) on Western blots with an antibody raised against the N-terminal amino acids 5–24 of the β-A subunit of 32 kDa bovine inhibin. No immunoreactive bands were detected, although with bFF a 14 kDa band emerged after reduction (results not shown). Furthermore, no staining was observed when the blots were incubated with preimmune serum (data not shown). Silver staining of these samples revealed that in the bioactive fraction besides a 31.7 kDa band two bands of 49 kDa en 21 kDa were present. After reduction of these samples the 31.7 kDa band disappeared and 18.4 kDa and 11.7 kDa bands emerged. Isoelectrofocussing and silver

staining of this sample revealed after silver staining one prominent protein with an apparent isoelectric point of 4.8 (results not shown).

Amino acid analysis. Purified fractions obtained after both methods 1 and 2 were subjected to N-terminal amino acid sequence analysis without further derivation. Most of the cycles revealed 2–4 amino acids. Table 2 lists the common amino acids which were found in the inhibin preparations obtained after both purification methods and the amino acids as predicted from rat cDNA sequences (Esch et al., 1987; Woodruff et al., 1987). In all cycles, with the exception of cycle 9, the amino acids found were consistent with the presence of an $\alpha\beta$-B inhibin dimer in rSCCM, while the amino acids specific for the β-A sequence were not detected. Glycine could not be detected with both methods in cycle 1 and 6, due to residual glycine from the SDS-PAGE buffer. Cysteines were not identified in cycle 4, 11 and 12 because no prior derivation was performed.

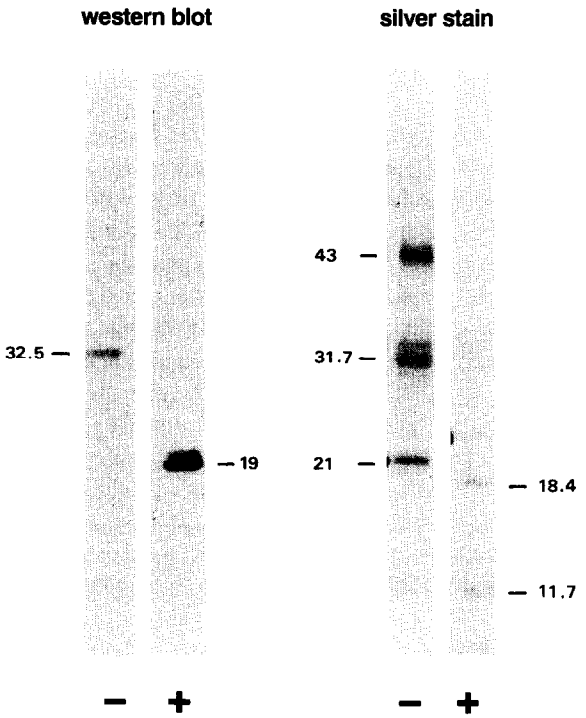

Fig. 5. Western blot analysis with an antiserum against the 22 N-terminal amino acids of the bovine inhibin α-subunit and silver staining of fraction 18 of Fig. 4. Fractions were analyzed by SDS-PAGE on 15% gels, with (+) or without (−) prior reduction with β-mercaptoethanol. Indicated molecular weights are based on the positions of molecular weight markers, which have not been indicated in this figure.

TABLE 2

AMINO ACID SEQUENCE ANALYSIS OF INHIBIN PREPARATIONS PURIFIED ACCORDING TO TWO METHODS

The N-terminal amino acid sequence of rat 30 kDa inhibin, derived from cDNA sequences (Esch et al., 1987 and Woodruff et al., 1987), is also presented for comparison.

Cycle	Amino acids detected with method 1 and 2	Predicted amino acids		
		α	β-A	β-B
1	S	S	G	G
2	Q, A, L	A	L	L
3	P, E	P	E	E
4	S	S	C	C
5	D, M	M	D	D
6	P	P	G	G
7	W, R	W	K	R
8	P, T	P	V	T
9	K, L	W	N	S
10	S, L	S	I	L
11	P	P	C	C
12	A	A	C	C
13	A	A	K	R
14	L, Q	L	K	Q
15	R, Q	R	Q	Q
16	L	L	F	F
17	F, L	L	F	F
18	Q	Q	V	I

Discussion

Although rSCCM is a rich source of inhibin, with an RSA of 17–21 compared with bFF, it is difficult to obtain sufficient material for purification procedures. This was the reason to use the double collagenase method for the isolation of Sertoli cells, instead of the isolation method used to obtain highly purified Sertoli cells, which gives lower yields (Toebosch et al., 1989). Although in this rSCCM some peritubular cell derived proteins will be present, the report that the peritubular cell derived factor PmodS stimulates inhibin production (Skinner et al., 1989), makes it also attractive to use the former method. Up to the fifth week of culture appreciable amounts of bioactive inhibin were produced after an initial rise in the second week.

The Amicon hollowfibre concentration system with a molecular weight cut-off at 10 kDa was a convenient way to concentrate/exchange large pools of rSCCM in a short period. Inhibin bioactivity was never detected in the < 10 kDa fractions, also not after concentration of this fraction using a membrane with a cut-off at 1 kDa and testing equivalents up to 6 ml 1–10 kDa rSCCM per 0.5 ml pituitary culture medium (data not shown). After concentration several purification steps have been explored. The Mono Q anion-exchange column resulted in inhibin preparations with high yields and a reasonable increase of RSA. When reversed-phase chromatography was performed directly on Amicon concentrated rSCCM, FSH-releasing activity eluting in front of inhibin was observed. After the Mono Q column this FSH-releasing activity was lost (results not shown).

In the first purification method after the Mono Q column a C1/C8 column was used, followed by SDS-PAGE. Amino acid sequence analysis revealed a heterogeneous preparation. With the second purification method, two reversed-phase chromatography steps at neutral and acid pH were used. With this method a low RSA was obtained compared with the result of the first method. Western blot analysis of the inhibin preparation purified with method 2 revealed that only 15 U of bioactive inhibin resulted in a signal which was stronger than 1000 U starting rSCCM. This indicates that bioactivity is less stable than immunoactivity, resulting in an underestimation of the RSA of the purified inhibin using method 2.

A 32 kDa protein with inhibin bioactivity was purified from rSCCM. This protein is composed of a 18.4 kDa α-subunit and 11.7 kDa β-B subunit linked by disulphide bond(s). The molecular weight of this purified inhibin preparation confirms the observation that in rat testis homogenates and rSCCM directly separated by SDS-PAGE only one form of 32 kDa bioactive inhibin was found (Grootenhuis et al., 1989). This makes it likely that the 32 kDa $\alpha\beta$-B inhibin molecule is the predominant form of inhibin in the rat testis, although it cannot be excluded completely that other forms of inhibin have been lost during the purification procedure. Also Bicsak et al. (1987) reported a 30 kDa inhibin form in rSCCM chromatographed under denaturing conditions on a Superose-12 gel permeation column. A similar molecular weight form of inhibin (32 kDa) was found in the conditioned medium of rat granulosa cells (Bicsak et al., 1986). Molecular weight estimations of rat testicular preparations using gel permeation chromatography under non-denaturing conditions revealed large differences (30–100 kDa) in molecular weight (Au et al., 1983; Noguchi et al., 1987; Sharpe et al., 1988), probably due to hydrophobic interactions. The high molecular weight form of inhibin, found after Lentil Lectin-Sepharose chromatography of purified rSCCM, which we reported earlier (de Jong et al., 1987), was probably due to a silver stain artefact at 65 kDa.

The molecular weight of inhibin and its subunits after reduction agrees well with the reports on purified 32 kDa ovarian inhibin (Ling et al., 1985; Miyamoto et al., 1985; Rivier et al., 1985; Fukuda et al., 1986; Robertson et al., 1986; Leverha et al., 1987). The amino acid sequence which was obtained after combination of the data for the two inhibin preparations shows that rat Sertoli cell inhibin is an $\alpha\beta$-B dimer. Additional evidence was obtained using an antiserum recognizing the β-A subunit of inhibin; using this antiserum no immunoreactive bands were detected on the Western blot. This agrees with the presence of mRNAs for the α- and β-B subunits of inhibin in rat Sertoli cells and the low or undetectable levels of β-A mRNA (Toebosch et al., 1988; Feng et al., 1989; Klaij et al., 1990). The only other reports on the characterization of male inhibin revealed a 30 kDa $\alpha\beta$-A dimer in ovine testicular lymph (Bardin et al., 1987; Vaughan et al., 1989).

The retention of inhibin bioactivity on Lentil Lectin-Sepharose indicates that rat inhibin is a glycoprotein as was reported for inhibin from other species. The isoelectric point of 32 kDa rat inhibin is similar to that reported by van Dijk et al. (1985) for bFF inhibin purified on a chromatofocussing column.

It is concluded that rat testicular inhibin is a 32 kDa $\alpha\beta$-B dimer. This contrasts sharply with the situation in follicular fluid where both $\alpha\beta$-A and $\alpha\beta$-B dimers were found with a number of molecular weight forms of inhibin (Ling et al., 1985; Robertson et al., 1985; Miyamoto et al., 1986; Grootenhuis et al., 1989).

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