Deglycosylated Human Follitropin: Characterization and Effects on Adenosine Cyclic 3′,5′-Phosphate Production in Porcine Granulosa Cells†

Francisco O. Calvo,‡ Henry T. Keutmann,§ Elizabeth R. Bergert,‡ and Robert J. Ryan*,‡

Department of Biochemistry and Molecular Biology, Mayo Medical School, Rochester, Minnesota 55905, and Endocrine Research Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

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ABSTRACT: Chemical deglycosylation of gonadotropins with hydrogen fluoride (HF) has facilitated the investigation of the structure–function relationship of the individual peptide and oligosaccharide moieties in the mechanism of hormone action. These studies have dealt almost exclusively with lutropin or human choriogonadotropin. We report here the chemical characterization and biological properties of deglycosylated human follitropin (degly hFSH). Results indicate that deglycosylation of hFSH by HF removes 89% of the total carbohydrate without disruption of the peptide chain or significant loss of amino acid residues. However, a change in the conformation of the molecule was observed by measurement of the far-ultraviolet circular dichroic spectrum. The degly hFSH showed a 44% reduction in binding when tested in a FSH radioimmunoassay utilizing a polyclonal antibody. Binding of the degly hFSH to FSH-responsive tissues showed that the altered hormone bound with equal or better avidity than the intact hormone while the association constants were approximately the same for both preparations. The degly hFSH alone did not stimulate the FSH-stimulatable adenylyl cyclase (AC) activity of cellular homogenates of small follicle porcine granulosa cells. Furthermore, degly hFSH was a potent antagonist of hFSH-stimulatable AC activity when coincubated with intact hFSH. In intact granulosa cells, both the hFSH and the degly hFSH stimulated cAMP production and release by these cells. However, the degly hFSH was one-tenth as effective as the intact hormone. Paradoxically, coincubation of the degly hFSH (up to 5-fold excess) with intact hFSH either at the same time or with a 10-min preincubation of the degly hFSH with the cells did not result in an inhibition of cAMP production in intact cells. Results support the notion that the carbohydrate moieties play an important role in the transduction and initiation of the biological response in hormone-responsive tissues. The poor antagonistic properties of degly hFSH in intact granulosa cells may be due to the presence of spare receptors, to a fast association and/or dissociation rate constant, or perhaps to the conformational change which has been observed.

Human choriogonadotropin (hCG),[1] equine choriogonadotropin (PMSG), lutropin (LH), follitropin (FSH), and thyrotropin (TSH) are glycoproteins with complex oligosaccharide chains which comprise 21–45% of the molecular mass (Pierce & Parsons, 1981; Sairam, 1983). Studies of the structure–function relationships of the glycoprotein hormones have been facilitated by the advent of the one-step chemical deglycosylation of proteins with hydrogen fluoride (HF). This chemical modification has given an insight into the role which the individual peptides and carbohydrate moieties play in the initiation of the biological responses.

Recent studies have indicated that the deglycosylated hormones bind better in radioreceptor assays than their intact counterparts. In contrast, they have a greatly diminished capacity for stimulation of cAMP production and steroid biosynthesis. In addition, the deglycosylated preparations are effective competitors of the intact hormones in in vitro bioassays (Chen et al., 1982; Manjunath et al., 1982a,b; Sairam & Manjunath, 1982; Ryan et al., 1982; Keutmann et al., 1983, 1985; Liu et al., 1984). These changes in the activity of the deglycosylated preparations have been ascribed to the loss of the N-linked carbohydrate chains. Indeed, recent work by Calvo and Ryan (1985) has shown that glycopeptides and oligosaccharides prepared from hCG and its subunits are able to effectively inhibit the stimulatory effects of intact hCG in the rat corpora lutea adenylyl cyclase (AC) system.

Recent studies which have probed the structure–function relationship of the glycoprotein hormones by utilizing the chemically modified analogues have dealt, almost exclusively, with LH or hCG [for a review, see Ryan & McIlroy (1982) and Sairam (1983)].

There are few studies which have been concerned with FSH (Giudice et al., 1978; Manjunath et al., 1982a; Sairam & Manjunath, 1982; Keutmann et al., 1985). This paper reports our findings on the chemical and biological features of the deglycosylation of highly purified human FSH.

Materials and Methods

Hormone Preparation. Purified human follitropin (hFSH, AFP 4161B) was a gift of The National Hormone and Pi-

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‡ Mayo Medical School.

§ Massachusetts General Hospital.

[1] Abbreviations: hCG, human choriogonadotropin; PMSG, equine choriogonadotropin; LH, lutropin; degly, deglycosylated; FSH, follitropin; TSH, thyrotropin; HF, hydrogen fluoride; AC, adenylyl cyclase; CD, circular dichroism; SFPGC, small follicle porcine granulosa cells; UV, ultraviolet; K_a, association constant; RIA, radioimmunoassay; RRA, radioreceptor assay; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MIX, 1-methyl-3-isobutylxanthine; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Cl_3CCOOH, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid.

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tuitary Program, NIADDK, Baltimore, MD. The biological potency of AFP 4161B is 4941 IU/mg (2nd International Reference Preparation of Human Menopausal Gonadotrophin).

Deglycosylation. The hydrogen fluoride procedure as used by Keutmann et al. (1983) was employed to deglycosylate the hFSH. Briefly, 10 mg of hFSH was dried in vacuo over P_2O_5 for 24 h. The hFSH was then transferred to a Toho polypropylene distillation apparatus (Toho Industries, Toho, Japan) and treated with 10 mL of anhydrous hydrogen fluoride (Matheson, East Rutherford, NJ) for 1 h at 0 °C. Anisole was omitted from the reaction. HF was removed under vacuum, and the granular, yellow-brown product was rinsed twice with cold anhydrous ether and then extracted with 10 mL of 10% acetic acid and centrifuged. The residue was reextracted several times with 10% acetic acid. The clear pooled extracts were lyophilized, redissolved in column buffer, and then desalted by gel filtration using a Sephadex G-100 (1.2 × 90 cm) column previously equilibrated with 0.2 M ammonium bicarbonate buffer, pH 8.7.

Carbohydrate and Amino Acid Analysis. The carbohydrate content of intact and deglycosylated hFSH was determined by gas chromatography after hydrolysis in 1 M methanolic HCl as described by Reinhold (1972) and Keutmann et al. (1983). The amino acid analysis was done on a Beckman Model 121MB automatic analyzer following acid hydrolysis in 6 N HCl at 100 °C for 24 h. For all data, the mass of hFSH or degly hFSH was based on amino acid analysis.

Circular Dichroic Studies. The UV circular dichroic spectra for intact and degly hFSH were measured on a Jasco J-500A spectropolarimeter (Japan Spectroscopic Co., Tokyo) with a light path of 1 mm for recording between 250 and 200 nm. Sixteen scans were made at a scanning speed of 50 nm/min. Intact hFSH and degly hFSH were dissolved in 50 mM phosphate buffer, pH 7.4.

Granulosa Cell Incubations. Granulosa cells from 1–3-mm follicles of ovaries from prepubertal pigs (SFPGC) were obtained from the local abattoir. The ovaries were transported on ice to the laboratory and dipped in an antibiotic solution consisting of 100 units of penicillin/mL, 250 ng of fungizone/mL, and 100 μg of streptomycin/mL (Grand Island Biological Co., Grand Island, NY), and the granulosa cells were aspirated aseptically. After being collected and pooled, the SFPGC were washed 3 times in sterile Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 15 mM HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid], 1 mM MIX (1-methyl-3-isobutylxanthine), 0.4% BSA (Sigma Chemical Co., St. Louis, MO), and 50 μg of gentamycin/mL (Grand Island Biological Co. Grand Island, NY), pH 7.4. The SFPGC were then counted with a hemocytometer and aliquotted into 12 × 75 mm disposable culture tubes which contained DMEM and either intact FSH or degly FSH, or a combination of the two, in a volume of 0.5 mL. Incubations were carried out in triplicate for 3 h in a shaking 37 °C water bath which was equilibrated with humidified 5% CO_2–95% O_2. The incubations were terminated by the addition of 0.5 mL of ice-cold 10% (w/w) trichloroacetic acid (Cl_3CCOOH) followed by sonication with a Model W-22OF cell disruptor (Heat Systems-Ultrasonics, Inc.) for 5 s at a setting of 3.5. After a 30-min incubation at 4 °C, the tubes were centrifuged at 1700*g* for 20 min. The pellets were used for DNA assay and the supernatants for the RIA of cAMP.

Assay Procedures. FSH binding to receptors was assayed as described by Andersen et al. (1983) utilizing membranes prepared from immature porcine or bovine testes. Assays were carried out in 50 mM TES [*N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid], 5 mM $MgCl_2$, 0.1 M sucrose, and 0.1% ovalbumin, pH 7.5 (Sigma Chemical Co., St. Louis, MO). The final assay volume was 100 μL. Nonspecific binding was measured by adding an excess (1000 IU/mL) of PMSG. In those experiments in which whole small follicle porcine granulosa cells were used for the measurement of binding activity, the cell pellet was reconstituted in assay buffer in an appropriate volume and assayed directly. The hFSH and degly hFSH were radioiodinated by the method of Greenwood et al. (1963) as modified by Lee and Ryan (1973).

Immunological cross-reactivity was tested in a double antibody hFSH radioimmunoassay as described by Faiman and Ryan (1967). The first antibody made against intact hFSH was generated in rabbits and was used at a final dilution of 1:10000. The sensitivity of the assay was 0.7 ng/tube.

Adenylyl cyclase activity of SFPGC in response to hFSH and degly hFSH was determined according to Lee (1978). The cells were washed twice in cold 0.9% NaCl containing 25 mM Tris buffer, pH 7.4, and resuspended in cold 10 mM Tris buffer, pH 7.5, containing 27% (w/v) sucrose and 1.0 mM EDTA. The cell suspension was homogenized in a 7-mL glass on glass Dounce homogenizer (Kontes Co., Evanston, IL) using 3 strokes with the tight pestle. Twenty-microliter aliquots of homogenate equivalent to 4×10^6 cells/tube (172 μg of protein) were used. The reaction was carried out for 20 min.

RIA of cAMP was performed on the Cl_3CCOOH supernatants (see above) by the method described by LaBarbera et al. (1982) after Cl_3CCOOH was removed by extraction with anhydrous diethyl ether equilibrated with water. The aqueous phase was then lyophilized and reconstituted to the original volume with 50 mM sodium acetate buffer, pH 6.5, and appropriately diluted in the assay buffer with 0.1% BSA to yield tracer displacements between 20% and 80%. Recoveries were determined by adding a constant amount of unlabeled cAMP to selected control tubes which were then treated as regular samples. Recoveries were routinely 80–85% and were used to correct for procedural losses. No cAMP was detected in control tubes which contained the culture medium alone. The cAMP used as standard was purchased from Sigma Chemical Co. and was used without further purification. The adenosine 3′,5′-cyclic phosphoric acid 2′-*O*-succinyl[^{125}I]iodotyrosine methyl ester, specific activity 2200 Ci/mM, used as the tracer, was purchased from New England Nuclear (Boston, MA). The cAMP antibody was generated in rabbits and used at a final dilution of 1:45000. The volume of incubation was 300 μL, and the assay sensitivity was 17 fmol (80% binding). The cAMP levels were calculated by using the C-fit algorithm described by Brooker et al. (1979) utilizing a Digital PDP 11/73 computer.

The DNA in the SFPGC pellets (see above) was quantitated by using the fluorescence method of Kissane and Robins (1958) and Lohr and Kovacic (1977) after the pellets were washed with 10 mM ethanolic potassium acetate and hydrolyzed in 0.5 M perchloric acid.

Results

Table I illustrates the amino acid composition of intact hFSH and degly hFSH. There was basically no difference in the amino acid composition of the two preparations. However, the carbohydrate analysis (Table II) shows that 89.38% of the total carbohydrate was removed by HF treatment. Only 75% of the *N*-acetylglucosamine was removed. This carbohydrate is N linked directly to asparagine and is not as susceptible to hydrolysis as the more distal carbohy-

Table I: Amino Acid Composition of Intact and Deglycosylated AFP 4161B Human Follitropin (Residues/Mole) after Acid Hydrolysis

amino acid	hFSH	degly hFSH	amino acid	hFSH	degly hFSH
aspartic acid	17.5	17.0	methionine	2.9	2.9
threonine	17.0	17.9	isoleucine	5.4	4.7
serine	15.3	16.8	leucine	11.1	10.4
glutamic acid	20.6	20.9	tyrosine	11.1	10.9
proline	ND[a]	ND	phenylalanine	9.1	8.7
half-cysteine	ND	ND	lysine	13.2	13.4
glycine	12.4	13.3	histidine	5.2	5.2
alanine	11.2	11.4	arginine	8.5	8.7
valine	9.0	9.6			

[a] ND for proline and half-cysteine indicates that these two amino acids, even though they were present, were not quantitated because of the nature of the procedure.

Table II: Carbohydrate Composition of Deglycosylated AFP 4161B Human Follitropin (Residues/Mole of hFSH) after Hydrogen Fluoride Treatment

	hFSH	degly hFSH
fucose	0	0
mannose	10.34	0.98
galactose	7.7	0
N-acetylgalactosamine	3.84	0.47
N-acetylglucosamine	13.56	3.38
sialic acid	10.06	0
	total 45.5	4.83

drates. Sephadex G-100 gel filtration analysis of the reduced and ^{14}C-carboxymethylated β subunit of hFSH and degly hFSH revealed that the β subunit of degly hFSH eluted as a single peak close to but slightly behind the position of the ^{14}C-carboxymethylated β subunit of intact hFSH, thus showing the absence of any peptide cleavages which may have been affected by the HF hydrolysis (data not shown). Previous experiments have shown that the α subunit is also not cleaved by the deglycosylation procedure (Keutmann et al., 1985).

The far-ultraviolet circular dichroic spectra of intact hFSH and deglycosylated hFSH indicate that a shift toward a more random structure of the degly hFSH has occurred due to the deglycosylation (Figure 1), the degly hFSH having a minimum at 206 nm and intact hFSH at 209 nm. There was no difference in the near-UV spectrum between the two preparations (data not shown). Figure 2 depicts the radioimmunoassay data of intact hFSH and degly hFSH utilizing a polyclonal antibody which is specific for hFSH. In this assay system, the degly hFSH showed a 44% reduction in binding to the hFSH antibody when compared to the intact hFSH, perhaps reflecting a change in the conformation of the molecule due to the deglycosylation procedure.

When the degly hFSH was tested in a RRA utilizing three different FSH-responsive tissues, it was found that the degly hFSH bound with equal or better avidity than the intact hormone. These data are shown in Table III in which the relative potencies of degly hFSH are compared with that of intact hFSH. There was a 3-fold increase in the ability of degly hFSH to bind to the FSH receptor of bovine testes when compared to the intact preparation. However, there was basically no difference in the association constants (K_a) for the intact hFSH between the immature porcine and bovine testes. The K_a for the whole small follicle porcine granulosa cells was less than that obtained from the testicular tissue.

The FSH-stimulatable adenylyl cyclase activity of membranes derived from SFPGC in response to intact and degly hFSH was also tested (Figure 3). Increasing concentrations

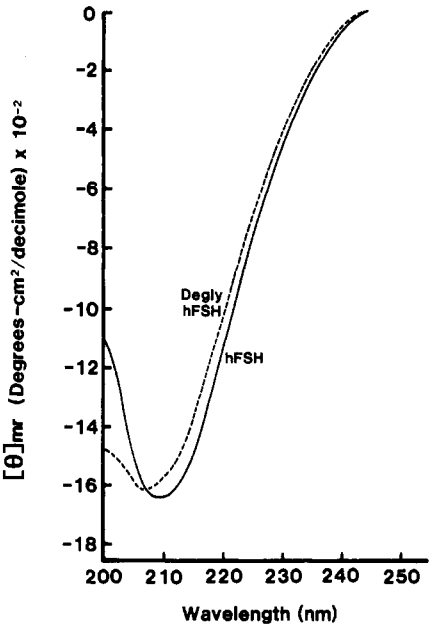

FIGURE 1: Far-ultraviolet circular dichroic spectra of intact hFSH and deglycosylated hFSH. The hormones were dissolved in phosphate buffer, pH 7.4, and the spectra derived as described under Materials and Methods.

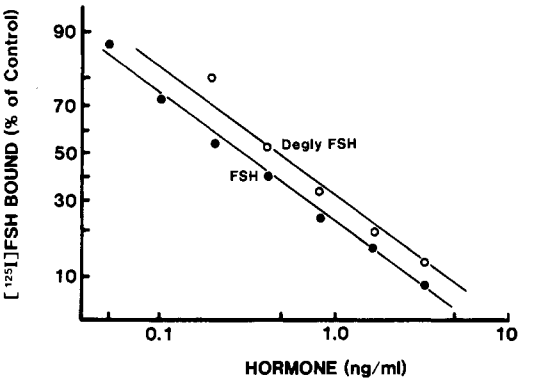

FIGURE 2: Follicle stimulating hormone double antibody radioimmunoassay of intact and deglycosylated hFSH. The first antibody, made against hFSH, was used at a final dilution of 1:10 000. The final volume of incubation was 500 μL. The second antibody was goat anti-rabbit plus a 1:60 dilution of normal rabbit serum. The antibody complex was precipitated by adding 500 μL of 4.8% polyethylene glycol 6000.

Table III: Potency Estimates and Association Constants (K_a) of Intact and Deglycosylated Human Follitropin in a FSH Radioreceptor Assay

	intact hFSH		deglycosylated hFSH	
tissue	potency (%)[a]	K_a (M^{-1})[b]	potency (%)	K_a (M^{-1})
immature porcine testes	100	5.89×10^9	121	3.9×10^9
immature bovine testes	100	6.52×10^9	309	3.1×10^9
whole small follicle, porcine granulosa cells	100	1.60×10^9	200	2.1×10^9

[a] Potency estimates were derived at 50% of control binding. The hormone mass, based on the amino acid composition, was converted to a molar concentration prior to the assessment of the potency estimates. The hormone weights were M_r 35 000 and M_r 28 000 for intact hFSH and deglycosylated hFSH, respectively. The estimation of the potency for the deglycosylated hFSH was performed with ^{125}I-hFSH as the radioactive ligand and unlabeled deglycosylated hFSH. [b] Estimations of the association constants by Scatchard analysis were performed by utilizing the homologous hormone. The standard error of the mean in each case was less than 10%.

Table IV: Effects of Coincubation of Deglycosylated Human Follitropin with Intact hFSH upon cAMP Production by Small Follicle Porcine Granulosa Cells[a]

concurrent incubation			10-min preincubation of degly hFSH[b]		
hormone (ng/mL)		cAMP	hormone (ng/mL)		cAMP
hFSH	degly hFSH	[pmol (μg of DNA)$^{-1}$ (3 h)$^{-1}$]	hFSH	degly FSH	[pmol (μg of DNA)$^{-1}$ (3 h)$^{-1}$]
5		84.4 ± 6.0	5		80.0 ± 8.0
5	5	80.2 ± 7.6	5	5	81.3 ± 13.3
5	10	120.8 ± 15.0	5	10	90.3 ± 9.2
	10	12.1 ± 0.3		10	ND[c]
20		741.3 ± 68.2	20		736.0 ± 47.1
20	30	790.6 ± 55.5	20	30	913.0 ± 118.1
20	100	766.1 ± 54.7	20	100	1332.3 ± 161.8
100		1536.2 ± 144.0			
100	100	1646.5 ± 246.7			
	100	25.8 ± 5.7			

[a] Incubations were carried out as described under Materials and Methods. Results are compiled from three separate experiments, each performed in triplicate. [b] The degly hFSH was preincubated for 10 min at 37 °C with the porcine granulosa cells prior to the addition of the intact hFSH. [c] ND, not done.

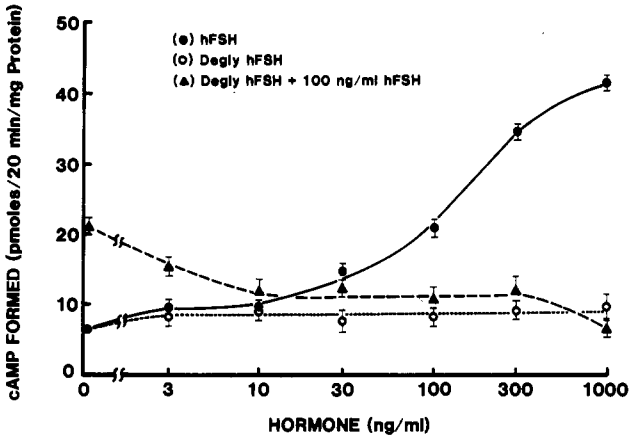

FIGURE 3: Adenylyl cyclase activity of cellular homogenates of small follicle porcine granulosa in response to increasing concentrations of hFSH, deglycosylated hFSH, or a combination of the two preparations. The cellular homogenization and adenylyl cyclase assay were performed as described under Materials and Methods. The cellular homogenate which was equivalent to 4×10^6 cells per tube (172 μg of protein) was preincubated at room temperature for 10 min with either the intact hFSH or the deglycosylated hFSH before the addition of the reaction mixture. The reaction was performed at 30 °C for 20 min. Each datum point represent the mean ± SEM of triplicate determinations from a single homogenate.

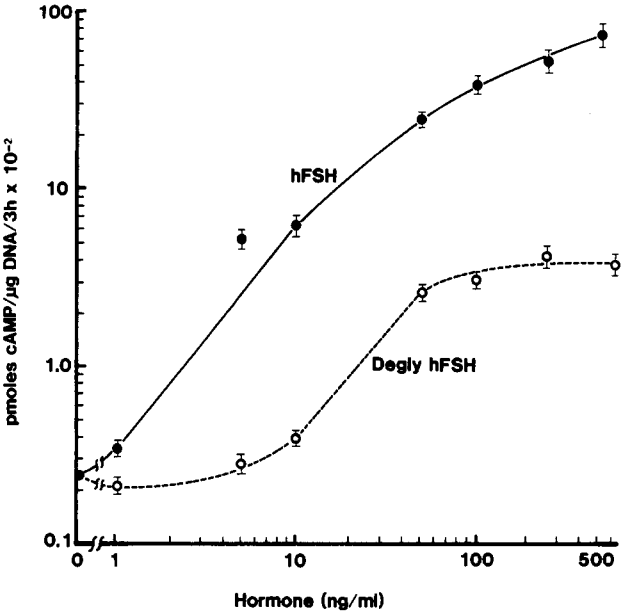

FIGURE 4: Effect of increasing concentrations of intact and deglycosylated hFSH (AFP 4161B) on cAMP production from small follicle porcine granulosa cells over a 3-h incubation at 37 °C. The isolation and preparation of the granulosa cells were as described under Materials and Methods. Approximately $(1.0 \times 10^7) \pm (0.96 \times 10^6)$ cells per incubation and were used. The incubations were terminated by the addition of an equal volume of 10% Cl_3CCOOH. The pellet, after sonication and centrifugation, was used for DNA determination and the supernatant for the determination of cAMP by RIA. Points represent the mean ± SEM of triplicate incubations and were repeated at least twice with essentially the same results.

of intact hFSH stimulated the cyclase enzyme in a dose–response fashion. The apparent K_m for FSH was calculated to be 92 ng/mL. However, the cAMP produced in response to increasing concentrations of degly hFSH was <1.5% that of the intact hormone. In addition, the degly hFSH was shown to be an effective antagonist of intact hFSH when increasing concentrations of degly hFSH were coincubated with 100 ng of intact hFSH/mL as shown in Figure 3.

Three-hour incubations of intact granulosa cells (SFPGC) with increasing concentrations of either intact hFSH or degly hFSH show that cAMP is produced in a dose-related manner in response to either preparation (Figure 4). The maximal stimulatory dose in each case was approximately 100 ng/mL, but the maximum response was approximately 14-fold greater for hFSH than for degly hFSH. The dose–response curve for degly hFSH was shifted to the right, and approximately 10 times as much degly FSH as intact FSH was required to increase cAMP production over basal secretion.

In contrast to the inhibitory effects of the degly hFSH on AC activity on SFPGC homogenates (see Figure 3), equal or greater concentrations of degly hFSH did not inhibit the cAMP production from intact SFPGC when coincubated with intact hFSH. These results are shown in Table IV. A concurrent incubation of 5 ng of hFSH/mL either with 5 or 10 ng/mL degly hFSH or with a 10-min preincubation of the degly hFSH prior to the addition of the intact hFSH did not suppress the stimulatory effects of the intact hormone on cAMP production, despite the fact that the degly hFSH had twice the affinity of the intact hFSH in the RRA (Table III). To preclude the possibility that the dosage of degly hFSH was too low to effectively compete with the intact hormone, we performed an additional set of experiments in which the final concentration of degly hFSH was 5 times that of the intact hFSH. In all cases, stimulation of cAMP by hFSH was not prevented by the degly hFSH even when preincubated with the granulosa cells. In fact, there seemed to be an additive effect of the degly hFSH because of the large concentrations of the deglycosylated hormone (Table IV) which also stimulate

cAMP production at higher doses (Figure 4).

Discussion

In earlier work, we observed no differences in the CD spectrum between native and deglycosylated hCG (Keutmann et al., 1983). In the present study, far-ultraviolet CD spectra of intact and degly hFSH indicated that there was a change in conformation after deglycosylation. The negative extremum of the degly hFSH was 206 nm compared to 209 nm for intact hFSH. Generally, the far-UV negative extrema for intact hLH, hCG, the deglycosylated counterparts of hLH and hCG, and hFSH have ranged from 206 to 215 nm with that of hFSH being 210 nm (Rathman & Saxena, 1974; Holladay & Puett, 1975; Puett et al., 1976; Giudice & Pierce, 1978; Giudice et al., 1978). Interestingly, the negative extrema of 206 nm for hLH and 207 nm for hCG have been ascribed to the N-acetylated carbohydrate groups such as *N*-acetylglucosamine and *N*-acetylgalactosamine. Removal of 89% of the total carbohydrate and 78% of the amino sugars may thus have contributed to this shift from 209 to 206 nm. However, Ishii and co-workers (Ishii et al., 1984) measured the conformational changes upon N-glycosylation of a variety of synthetic peptides and concluded that there was no interaction between the carbohydrate moiety and the peptide backbone. Even though a glycosylated synthetic peptide does not exhibit the same conformation as a glycosylated protein, the point to consider is that the carbohydrate moiety did not affect the conformation of the peptide. However, an increase in the rigidity of the peptide was noted. A decrease in the tryptophan fluorescence intensity spectra of degly ovine FSH was observed by Manjunath et al. (1982) even though the peptide chain had not been disrupted. This decrease may be attributed to a conformational change of the degly ovine FSH that would make the tryptophan less accessible to fluorescence excitation.

In terms of immunological activity, the change in the conformation was accompanied by a 44% decrease in antibody recognition of degly hFSH when compared with intact hFSH. This conformational effect was previously demonstrated by Keutmann et al. (1985), who tested the immunological activity of the degly hFSH (same preparation as used here), the degly hFSH α subunit, and the intact α subunit of hFSH with a monoclonal antibody generated against intact hFSH. The affinity of this antibody is dependent on the conformation of the α subunit in the intact hFSH. The reactivity of degly hFSH was diminished to 2% when compared with the intact hFSH, while that of degly α was increased by 7.5-fold. Furthermore, in assays using a region-specific polyclonal antibody to reduced and carboxymethylated α subunit, deglycosylation of hFSH increased the reactivity 7.5-fold over that of the untreated hormone (Keutmann et al., 1985). These results argue for a change in conformation of the α subunit of hFSH after deglycosylation.

As shown previously with ovine FSH (Manjunath et al., 1982a), ovine LH (Manjunath et al., 1982b), and hCG (Keutmann et al., 1983), the HF treatment did not result in peptide chain cleavage or loss of amino acid residues (Table I). Thus, direct damage to the peptide chain appears not to account for the observed conformational changes.

This change in the conformation of degly hFSH did not have any deleterious effect in terms of receptor binding to immature bovine and porcine testis and intact SFPGC. The binding was either the same or better than the intact hFSH as has been shown by other investigators for ovine FSH, degly hCG, and degly ovine LH (Manjunath et al., 1982a,b; Chen et al., 1982; Keutmann et al., 1983).

The effects of FSH on the stimulatable AC of hormone-responsive tissues are well documented (Lee, 1978; Lindsay & Channing, 1979). Our studies with the FSH-stimulatable AC system of SFPGC indicate similar kinetics as those found by Lee (1978). The concentrations of hFSH leading to half-maximal stimulation (apparent K_m) were 92 ng/mL which is less than that reported by Lee (1978). The apparent V_{max} was approximately the same as that reported earlier (Lee, 1978).

Degly hFSH did not stimulate the AC system of granulosa cell homogenates. It is also a potent antagonist of AC activation; 10 ng of degly hFSH/mL completely abolished the agonistic effects of 100 ng of hFSH/mL. The lack of stimulation by deglycosylated hormones has been reported previously (Ryan & McIlroy, 1983; Sairam, 1983). However, in terms of AC activation, this is the first report which addresses the antagonistic effects of degly hFSH on cAMP biosynthesis.

In contrast, as seen in Figure 4, in intact granulosa cells both the degly hFSH and the intact hFSH elicited the production of cAMP in a dose-related fashion. However, the maximal response of the degly hFSH was markedly attenuated, and 10 times more degly hFSH was needed to increase cAMP production over basal secretion. Similar results have been reported by Liu et al. (1984) with HF-treated ovine LH in rat Leydig cells. Governman et al. (1982) and Moyle et al. (1975) also reported an increase in steroid production with enzymatically deglycosylated hCG in mouse Leydig tumor cells and rat Leydig cells.

In intact granulosa cells, we were not able to demonstrate any inhibition of cAMP production by degly hFSH. These results are at variance with Sairam and Manjunath (1982), who found degly ovine FSH to be a potent antagonist of cAMP production in rat seminiferous tubules. Liu and co-workers (Liu et al., 1984) found that degly ovine LH inhibited testosterone production in rat Leydig cells only at concentrations 10-fold higher than that of intact LH. To preclude the possibility that our preparation contained some partially deglycosylated or intact hFSH which may have decreased the antagonistic effect, we chromatographed the degly hFSH on concanavalin A coupled to Sepharose 4B. About 2% of the preparation bound to concanavalin A. Therefore, neither the poor antagonistic properties of degly hFSH nor the stimulatory effect on cAMP production can be ascribed to contamination with intact FSH.

The reason for the poor antagonistic properties of degly hFSH in intact cells is not readily apparent; however, some explanations are possible. First, the reason we did not see an inhibition of cAMP release in intact SFPGC when coincubated with intact hFSH may be related to the fact that there are more FSH receptor sites in the intact granulosa cells than are necessary for a maximal response, the so-called spare receptors (Catt & Dufau, 1973). The fact that not all the receptor sites need to be filled is of importance because even 1% receptor occupancy by intact hFSH may be enough to override any inhibitory effect of degly hFSH. Indeed, very small amounts of cAMP seem to be required to initiate steroidogenesis (Dufau et al., 1977). Also, recent studies by one of us (H. T. Keutmann, unpublished results) have established that degly TSH is a poor antagonist of thyroid cell function so this lack of antagonism may not be an isolated effect.

Second, the difference between our results and those of Sairam and Manjunath (1982) may lie in the fact that we used porcine granulosa cells and their studies employed collagenase-dispersed immature rat testicular seminiferous tubules. Also, their incubations were carried out for 30 min, and ours

were for 3 h. Perhaps degly FSH and intact hormone differ in their kinetics for binding to receptor. Liu et al. (1984) and Ryan (unpublished results) have shown that the association rate constants for degly ovine LH and degly hCG are faster than those of the intact hormones, with the off rate being about the same for the ovine LH. If a fast association rate constant is a general property of deglycosylated hormones, perhaps the degly hFSH also has a fast off rate which is expressed as a lack of antagonistic activity in intact cells.

In previous studies (Keutmann et al., 1983; Calvo & Ryan, 1985) of degly hCG, we concluded that binding of the peptide portion of the hormone to its receptor was insufficient for activation of the cyclase enzyme and that binding of the carbohydrate portion of the hormone to a membrane lectin was required for biological activity. These conclusions were possible because there was no change in the primary or secondary structure of degly hCG although more recent studies (Keutmann et al., 1985) indicate a change in tertiary structure. Because of obvious changes in the secondary and tertiary structure of degly FSH, we cannot reach a similar conclusion for FSH. The poor ability of degly FSH to stimulate the cyclase enzyme could be due to the lack of carbohydrate or the change in the conformation of the peptide.

Registry No. cAMP, 60-92-4; adenylyl cyclase, 9012-42-4.

REFERENCES

Andersen, T. T., Curatolo, L. M., & Reichert, L. E., Jr. (1983) *Mol. Cell. Endocrinol. 33*, 37–52.

Brooker, G., Harper, J. F., Terasaki, W. L., & Moylan, R. D. (1979) *Adv. Cyclic Nucleotide Res. 10*, 1–33.

Calvo, F. O., & Ryan, R. J. (1985) *Biochemistry 24*, 1953–1959.

Catt, K. J., & Dufau, M. L. (1973) *Nature (London), New Biol. 244*, 219–221.

Chen, H.-C., Shimohigashi, Y., Dufau, M. L., & Catt, K. J. (1982) *J. Biol. Chem. 257*, 14446–14452.

Dufau, M. L., Tsuruhara, T., Horner, K. A., Podesta, E., & Catt, K. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3419–3423.

Faiman, C., & Ryan, R. J. (1967) *J. Clin. Endocrinol. Metab. 27*, 444–447.

Giudice, L. C., & Pierce, J. G. (1978) in *Structure and Function of the Gonadotropins* (McKerns, K. W., Ed.) pp 81–110, Plenum Press, New York.

Giudice, L. C., Pierce, J. G., Cheng, K.-W., Whitley, R., & Ryan, R. J. (1978) *Biochem. Biophys. Res. Commun. 81*, 725–733.

Goverman, J. M., Parsons, T. F., & Pierce, J. G. (1982) *J. Biol. Chem. 257*, 15059–15064.

Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114–118.

Holladay, L. A., & Puett, D. (1975) *Arch. Biochem. Biophys. 171*, 708–720.

Ishii, H., Inoue, Y., & Chujo, R. (1984) *Int. J. Pept. Protein Res. 24*, 421–429.

Keutmann, H. T., McIlroy, P. J., Bergert, E. R., & Ryan, R. J. (1983) *Biochemistry 22*, 3067–3072.

Keutmann, H. T., Johnson, L., & Ryan, R. J. (1985) *FEBS Lett. 185*, 333–338.

Kissane, J. M., & Robins, E. C. (1958) *J. Biol. Chem. 233*, 184–188.

LaBarbera, A. R., Bergert, E. R., & Ryan, R. J. (1982) *Endocrinology (Baltimore) 111*, 1897–1903.

Lee, C. Y. (1978) *Endocrinology (Baltimore) 103*, 1153–1158.

Lee, C. Y., & Ryan, R. J. (1973) *Biochemistry 12*, 4609–4619.

Lindsey, A. M., & Channing, C. P. (1979) *J. Endocrinol. 80*, 9–20.

Liu, W.-K., Young, J. D., & Ward, D. N. (1984) *Mol. Cell. Endocrinol. 37*, 29–39.

Lohr, D., Kovacic, R. T., & Van Holde, K. E. (1977) *Biochemistry 16*, 463–477.

Manjunath, P., Sairam, M. R., & Sairam, J. (1982a) *Mol. Cell. Endocrinol. 28*, 125–138.

Manjunath, P., Sairam, M. R., & Schiller, P. W. (1982b) *Biochem. J. 207*, 11–19.

Moyle, W. R., Bahl, O. P., & März, L. (1975) *J. Biol. Chem. 250*, 9163–9175.

Pierce, J. G., & Parsons, T. F. (1981) *Annu. Rev. Biochem. 50*, 465–495.

Puett, D., Nureddin, A., & Holladay, L. A. (1976) *Int. J. Pept. Protein Res. 8*, 183–191.

Rathman, P., & Saxena, B. B. (1974) in *Gonadotropins and Gonadal Function* (Moudgal, N. R., Ed.) pp 23–41, Academic Press, New York.

Reinhold, V. N. (1972) *Methods Enzymol. 25*, 244–249.

Ryan, R. J., & McIlroy, P. J. (1983) in *Role of Peptides and Proteins in Control of Reproduction* (McCann, S. M., & Dhindsa, D. S., Eds.) pp 133–145, Elsevier, New York.

Ryan, R. J., McIlroy, P. J., Ratanabanangkoon, K., & Keutmann, H. (1982) in *Intraovarian Control Mechanisms* (Channing, C. P., & Segal, S. J., Eds.) pp 321–330, Plenum Press, New York.

Sairam, M. R. (1983) *Horm. Proteins Pept. 11*, 1–79.

Sairam, M. R., & Manjunath, P. (1982) *Mol. Cell. Endocrinol. 28*, 139–150.