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Chemical Synthesis of Peptide Fragments of the Hormone-Specific β-Subunit of Human Follicle-Stimulating Hormone†

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ABSTRACT: In order to determine the specific antigenic determinants of human follicle-stimulating hormone (hFSH), hFSH-β peptides with amino acid residues 33-49 (V2), 95-118 (V3), 76-118 (V3 + $^1/_2$C2), 1-33 (V1 + C1), 22-33 ($^1/_2$C1), and 95-107 (V3 + $^1/_4$C2) according to the nomenclature of Stewart and Stewart [Stewart, M., & Stewart, F. (1977) *J. Mol. Biol. 116*, 175] as well as additional peptides with the residues 93-107, 91-107, 89-107, 87-107, and 85-107 were chemically synthesized. The peptides were examined in radioimmunoassay systems of FSH, luteinizing hormone (LH), or human chorionic gonadotropin (hCG). V3 + $^1/_2$C2 and V1 + C1 showed immunological activity, whereas the other peptides did not. Antibodies were raised in rabbits against these peptides and examined for specific binding with hFSH, LH, thyroid-stimulating hormone (TSH), and hCG. V3 + $^1/_2$C2 as well as V1 + C1 produced antisera, which specifically bound hFSH, hLH, and hTSH, indicating that the amino acid sequences contained in hFSH-β peptides V3 + $^1/_2$C2 and V1 + C1 share common antigenic sites with hLH and hTSH. Antisera were produced in rabbits against hFSH-β, against reduced and S-aminoethylated hFSH-β (AE-FSH-β), and against AE-FSH-β coupled to hemocyanin. Reduced and S-aminoethylated β-subunit of FSH-β coupled with hemocyanin produced antisera in rabbits that specifically bound only hFSH and not hLH, hTSH, or hCG.

The primary amino acid sequences of the α- and β-subunits of human pituitary follicle-stimulating hormone (hFSH), luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG) have been elucidated (McKerns, 1978). The amino acid sequences of the α-subunit of these glycoprotein hormones are identical, whereas those of the hormone-specific β-subunits are different. The structural similarities among these hormones do not permit an easy recognition of the hormone-specific antigenic sites and create difficulties in the production of specific antisera by the use of either native hormones or their hormone-specific β-subunits as antigens. The importance of the conformation of the antigen in producing specific antibodies has been well recognized. For example, antibodies against the C-terminal peptides of the hCG-β subunit show specific binding with hCG but fail to neutralize the biological activity of the intact hormone in vivo and thus fail to compete with hCG at the receptor site (Louvet et al., 1976). Therefore, on the basis of (1) the immunological activity of hFSH peptides recovered during amino acid sequence determination, (2) the location of the disulfide bonds (Rathnam et al., 1982), (3) the location of the carbohydrate moieties (Tolvo et al., 1982), (4) the location of the "variable" and "constant" regions (Stewart & Stewart, 1977), and (5) the location of the disulfide bonds that are essential for the molecular conformation, we selected various regions of the hFSH-β amino acid sequence for chemical synthesis. The synthetic peptides were used as antigens to produce antibodies in rabbits. The synthetic peptides and antibodies against them were examined for immunological

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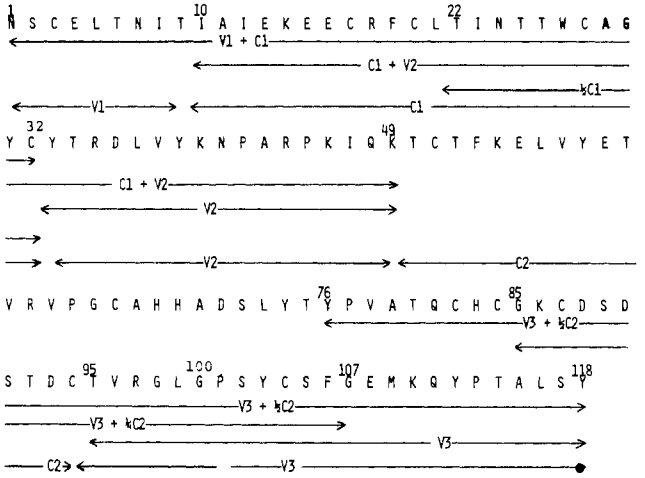

FIGURE 1: Synthetic fragments of hFSH-β. The following single-letter codes were used for amino acids: S, Ser; K, Lys; E, Glu; P, Pro; L, Leu; R, Arg; C, Cys; I, Ile; N, Asn; A, Ala; T, Thr; B, Asx; V, Val; G, Gly; Y, Tyr; M, Met; Q, Gln;D, Asp; F, Phe; H, His; W, Trp.

activity in specific radioimmunoassays of hFSH, LH, TSH, and hCG.

In this study, we have also modified hFSH-β by reduction and S-aminoethylation followed by coupling with hemocyanin. HFSH-β, reduced and S-aminoethylated hFSH-β (AE-FSH-β), and AE-FSH-β coupled to hemocyanin were injected into rabbits, and the sera, collected periodically, were tested for binding with hFSH, hLH, and hTSH.

Experimental Procedures

An analysis of the amino acid sequences of the "hormone-specific" β-subunits (Stewart & Stewart, 1977) has indicated that the β-subunits of hFSH, hLH, hTSH, and hCG share three "variable" or heterologous (V1, V2, and V3) and two "constant" or homologous (C1 and C2) regions. On the basis of the nomenclature proposed by Stewart & Stewart (1977), hFSH-β peptides, containing the regions of V2 (residues 33–49), V3 (residues 95–118), V3 + $^1/_2$C2 (residues 76–118), V1 + C1 (residues 1–33), $^1/_2$C1 (residues 22–33), C1 (residues 10–33), V3 + $^1/_4$C2 (residues 95–107), and other intermediates, viz., residues 93–107, residues 91–107, residues 89–107, residues 87–107, and residues 85–107, were synthesized (Figure 1).

Solid-Phase Peptide Synthesis. The fragments V2 (residues 33–49), V3 (residues 95–118), and V3 + $^1/_2$C2 (residues 76–118) were synthesized by the use of chloromethyl-resin (Merrifield, 1964). In order to achieve improved yields, the other fragments were synthesized by the use of PAM-resin (Mitchell et al., 1976). The PAM-resin was also acetylated after being coupled with the C-terminal amino acid in order to block the free reactive groups. The peptides were synthesized in a Beckman Model 990 peptide synthesizer, by using the *o*-bromocarbobenzoxy derivatives (BrCB) of L-tyrosine, *O*-benzylthreonine, *N*-tosyl-L-arginine, L-aspartic acid β-benzyl ester, L-leucine, L-valine, L-asparagine *p*-nitrophenyl ester, L-proline, L-alanine, L-isoleucine, and L-glutamine *p*-nitrophenyl ester. The C1 (*Z*)-BrCB-Lys was preferred, since it yields least degree of branching. ACM-cysteine was used since it is stable to HF cleavage (Ontjes et al., 1978) and thus allowed the purification of the peptide prior to deblocking of the Cys residues. The BrCB-amino acids were obtained from Beckman Instruments and were coupled by the DCC procedure (Stewart & Young, 1969) routinely used with the Beckman synthesizer. In the case of asparagine and glutamine, an active ester coupling method was used (Bodanszky & Sheehan, 1959). After the coupling of each amino acid, an aliquot of the resin was hydrolyzed for 3 h at 140 °C with concentrated HCl–propionic acid (1:1 v/v) and subjected to amino acid analysis to confirm the coupling of the amino acid. If necessary, the amino acid was recoupled to achieve the proper sequence. After synthesis, the peptides were deblocked and cleaved off the resin with hydrogen fluoride and anisole as scavenger by the use of a HF trap (Peninsula Laboratories, CA) at 0 °C for 1 h. The cleaved peptides were extracted from the resin with 0.1 M acetic acid and lyophilized. The peptides were redissolved in 0.2 M acetic acid and purified in aliquots of 100 mg by gel filtration on a 1.5 × 250 cm column of Sephadex G-50 (superfine). The fractions obtained from the Sephadex G-50 column were pooled according to the absorbance at 206 nm. The aliquots were subjected to amino acid analysis. The fraction containing the major amount of the synthetic peptide was further fractionated by either ion-exchange chromatography or by HPLC. The purity of the peptides was validated by (1) amino acid analysis, (2) thin-layer chromatography on MN-300 cellulose plates, (3) analytical electrophoreses on paper at different pHs, (4) N- and C-terminal amino acid determinations, and (5) sequence determination by Edman degradations and subtractive amino acid analyses of the peptide.

Purification of Synthetic Peptides. (A) Ion-Exchange Chromatography. The peptides were further purified by ion-exchange chromatography on a 1 × 100 cm column of Technicon peptide resin. A nine-chamber gradient elution of pyridine–acetate buffers was used to elute the column. A 50-μL aliquot of each fraction was reacted with ninhydrin. Fractions containing the synthetic peptides were pooled and further purified by high-voltage paper electrophoreses at pHs 6 and 3.5. The electrophoreses were performed as described earlier (Rathnam & Saxena, 1975). The fractions containing the synthetic peptides were eluted from the paper and characterized.

(B) High-Pressure Liquid Chromatography. High resolution was achieved of some peptides by HPLC on a C-18 column and buffers containing acetonitrile (buffer A was 0.05% TFA in water; buffer B was 0.035% TFA in 70% acetonitrile) for elution (Waters Associates model). A flow rate of 2 mL/min was maintained, and the eluate was monitored for absorbance at 206 nm. Fractions were collected, pooled according to their absorbance and analyzed for amino acid compositions to locate the synthesized peptides.

Characterization of Synthetic Peptides. The synthetic peptides were assayed in radioimmunoassays of FSH, LH, and hCG (Saxena, 1981).

Absorption of hFSH-β Antiserum with Synthetic Peptides. In order to test whether any of the synthesized peptide will inhibit or compete with the binding of hFSH-β to its antiserum, aliquots of 50 μL of hFSH-β antiserum at a dilution of 1:10 000 were preincubated for 2 h at 37 °C with 1, 5, and 10 nmol of each of the synthetic peptides, namely, ACM-formyl-$^1/_2$C1, V2, ACM-V3 (residues 95–118), ACM-V3 (residues 95–107), ACM-(V3 + $^1/_4$C2) (residues 91–107), and V3 + $^1/_2$C2). After the preincubation period, ^{125}I-hFSH-β (17 000 cpm) was added to the tubes, and the incubation was extended for 16 h. The degree of binding was determined. Control tubes without peptide were included in the experiment.

Reduction and S-Aminoethylation of hFSH-β. Three milligrams of hFSH-β was reduced and S-aminoethylated by the same procedures described by us earlier (Rathnam & Saxena, 1975). The reduced and S-aminoethylated FSH-β was coupled to hemocyanin, as follows: 2.2 mg of the reduced

Table I: Characterization of a Synthetic Peptide and Sequential Edman Degradation and Amino Acid Analyses

synthetic peptide	V3 + $^1/_2$C2
C-terminal amino acid[a]	tyrosine (0.5 nmol released/nmol of peptide)
N-terminal amino acid[b]	tyrosine
sequence	Tyr^{76}-Pro-Val-Ala...Tyr

	nmol/nmol of peptide			
amino acid	native	cycle 1	cycle 2	cycle 3
Asp	2.8	2.7	2.6	2.6
Thr	3.7	3.8	3.6	3.5
Ser	4.8	4.6	4.8	4.6
Glu	2.8	2.7	2.7	2.7
Pro	2.8	2.6	1.9	1.8
Gly	3.5	3.6	3.7	3.8
Ala	1.7	1.8	1.8	1.5
Cys				
Val	1.6	1.5	1.3	0.9
Mer	0.8	0.8	0.7	0.7
Leu	1.6	1.7	1.5	1.5
Tyr	4.0	3.5	3.2	3.2
Phe	1.0	1.0	1.0	1.0
His	0.8	0.7	0.6	0.6
Lys	1.8	1.5	1.4	1.4
Arg	0.8	0.6	0.6	0.5

[a] Digestion for 3 min with carboxypeptidases A and B (Fraenkel-Conrat et al., 1958). [b] Dansyl procedure (Gray & Hartley, 1963).

and S-aminoethylated FSH-β (AE-FSH-β) was coupled to 0.4 mL of a 5 mg/mL solution of hemocyanin (Pacific Biomarine, Venice, CA, in 0.5% K_2CO_2), by shaking the mixture at room temperature for 30 min. The reaction was terminated by dialysis against water for 24 h.

Production of Antibodies to Synthetic Peptides and to hFSH-β, AE-FSH-β, and AE-FSH-β Coupled to Hemocyanin. In order to avoid metabolic clearance due to the low molecular weight and to produce antibodies, the synthetic peptides were coupled to bovine serum albumin (Goodfriend et al., 1964) and injected into rabbits in a suspension of Freund's adjuvant, twice weekly, with 50 μg/mL per immunization.

HFSH-β, the reduced and S-aminoethylated hFSH-β (AE-FSH-β), and the AE-FSH-β coupled to hemocyanin were injected twice weekly into rabbits in a suspension of Freund's adjuvant with 50 μg/mL per immunization.

Periodically, the rabbits were bled, and the sera were examined for antibodies in a radioimmunoassay system of hFSH. The antisera were also examined for cross-reaction with LH and TSH by determining the specific binding to iodinated LH and TSH in the respective RIA systems.

Results and Discussion

Yield and Characterization of Synthetic Peptides. The synthesized peptides were characterized by amino acid analyses. The N- and C-terminal amino acid and the sequence determinations indicated the correct synthesis of the peptides in the manner shown for V3 + $^1/_2$C2 in Table I. The yield of the chemically synthesized peptides was low, being only <2–5 μmol after extensive purification; however, it was sufficient for the production of antibodies.

Immunological Activity of the Synthetic Peptides. Only the synthetic peptides ACM-(V3 + $^1/_2$C2) and ACM-formyl-$^1/_2$C1 exhibited immunological activity when examined in specific radioimmunoassays of FSH, LH, and hCG. ACM-(V3 + $^1/_2$C2) yielded 3, 1.8, and 1 ng of hormonal activity/100 nmol of peptide in FSH RIA, LH RIA, and hCG RIA, respectively, whereas the ACM-formyl-$^1/_2$C1 contained 3 ng of FSH activity/100 nmol of peptide. The rest of the peptides did not yield detectable levels of immunological activities.

Table II: Absorption of hFSH-β Antiserum with Synthetic Peptides

	% inhibition of hFSH-β binding at a dose of [a]		
fragment	1 nmol	5 nmol	10 nmol
ACM-formyl-$^1/_2$C1	0.9	3.5	7.7
V2	0	0	0
ACM-V3 (residues 95–118)	0	0	0
ACM-V3 (residues 95–107)	0	0	0
ACM fragment (residues 91–107)	0	0	0
ACM (V3 + $^1/_2$C2)	3.0	5.2	7.4

[a] Average of two determinations.

Table III: Characterization of Antisera to ACM-(V3 + $^1/_2$C2) and to ACM-formyl-(V1 + C1)[a]

antisera (final dilution 1:100)	displaced with	% specific binding to ^{125}I-FSH-β
ACM-(V3 + $^1/_2$C2)	FSH[b]	89
	FSH-β[b]	93
	(V3 + $^1/_2$C2)[c]	24
	(V3 + $^1/_2$C2)[d]	55
	hCG[c]	16
	hLH[b]	94
	hTSH[b]	91
ACM-formyl-(V1 + C1)	FSH[b]	71
	LH[b]	2
	TSH[b]	9

[a] The sera from rabbits injected with the other synthetic peptides did not show binding to either ^{125}I-FSH or ^{125}I-FSH-β up to dilutions of 1:10. [b] 1 μg/mL. [c] 10 μg/mL. [d] 100 μg/mL.

Absorption of hFSH-β Antiserum with Synthetic Peptides (Table II). Out of the six peptides examined in this experiment, the ACM-(V3 + $^1/_2$C2) (residues 76–118) and the ACM-formyl-$^1/_2$C1 (residues 22–33) showed progressive inhibition of hFSH binding to the antiserum with increasing concentration of the synthetic peptides. The ACM-V3 by itself did not show any inhibition of the binding, indicating that the C2 region of the ACM-(V3 + $^1/_2$C2) fragment (residues 76–95) was an essential component of the antigen to exhibit inhibition in the competitive protein binding RIA system. Similarly, the ACM-formyl-$^1/_2$C1, containing the constant region C1, indicated significant inhibition. These observations confirm the proposition that the constant regions in the β-subunits are involved in subunit–subunit interaction (Stewart & Stewart, 1977). Since the constant regions, due to their interaction with α-subunit, may be more superficial, an increased binding of the C1- and C2-containing peptides with the corresponding regions of the hFSH-β antiserum should be expected.

Production of Antisera to Synthetic Peptides. The ACM peptides (V3 + $^1/_2$C2) and ACM-(V3 + $^1/_4$C2) with cysteine residues blocked by the ACM-group produced antisera that bound hFSH, LH, and TSH (Table III), indicating that (1) common antigenic determinants to these hormones were contained in these regions and (2) the disulfide bonds in the $^1/_2$C2 or $^1/_4$C2 regions may not be essential for the immunological reactivity. Substitutions and/or deletions of the homologous amino acid residues in these regions, especially in the identical regions, namely, C1 and C2, may increase the specificity of binding to only one of the hormones.

Production of Antisera to hFSH-β, AE-FSH-β, and AE-FSH-β Coupled to Hemocyanin. Antiserum to hFSH-β and AE-FSH-β showed 13 and 6% specific binding to ^{125}I-hFSH-β; however, it cross-reacted 30–60% with ^{125}I-LH and ^{125}I-TSH. Antiserum produced to AE-FSH-β coupled to hemocyanin,

however, showed 22.5 and 9.5% specific binding to ^{125}I-FSH-β at dilutions of 1:10 and 1:20, respectively, but showed no cross-reaction with ^{125}I-LH or ^{125}I-hCG. This suggests a blocking of the LH and TSH binding sites in the antisera by hemocyanin. A similar effect has been reported by hemocyanin with antisera to hCG-β (Bahl et al., 1980). Hence, the AE-FSH-β coupled to hemocyanin may be a useful peptide to produce specific poly- and/or monoclonal antibodies against hFSH for use in RIA or in fertility control. Antibodies raised to a chemically modified intact hFSH-β subunit are also likely to produce antibodies that may reflect the conformational antigenicity of the hormone-specific subunit and thus neutralize the biological activity of the hormone by competing for binding of the hormone at the receptor site. Such antibodies may yield a bioimmuno coefficient of correlation close to unity and avoid nonspecific immunological interference in the measurement of the biologically active form of the hormone.

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Registry No. V2, 94294-36-7; V3, 94323-93-0; V3 + $^1/_2$C2, 94347-77-0; ACM-formyl-($^1/_2$C1), 94294-37-8; ACM-formyl-(V1 + C1), 94458-27-2; human follicle-stimulating hormone, 9002-68-0.

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