Isolation and Characterization of the Subunits of Bovine Follitropin

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Highly purified bovine follitropin was dissociated into its α - and β -subunits after treatment with 1M-propionic acid. The dissociated subunits were fractionated by chromatography on DEAE-cellulose and further purified by gel filtration on Sephadex G-100. The isolated α - and β -subunits were biologically inactive, but their recombinants regenerated 80% of the follitropin activity. The α -subunit of bovine follitropin recombined with the β -subunits of bovine lutropin and thyrotropin to regenerate 70% of lutropin and 50% of thyrotropin activities respectively. The β -subunit of bovine follitropin recombined with the α -subunit of either bovine lutropin or thyrotropin to regenerate about 75% of follitropin activity. Recombinations were monitored by specific radioligand-receptor assays and polyacrylamide-gel electrophoresis. The elution volumes of the α - and β -subunits of bovine follitropin after gel filtration on Sephadex G-100 were almost identical. The amino acid composition of bovine follitropin- α was low in histidine, arginine, isoleucine and leucine, but relatively high in lysine, threonine and glutamic acid. The bovine follitropin- β contained one methionine residue and low amounts of histidine and phenylalanine, but relatively high in aspartic acid, threonine and glutamic acid. The *N*-terminal residues of the α - and β -subunits of bovine follitropin were identified to be phenylalanine and glycine respectively.

The subunits of human (Rathnam & Saxena, 1975; Shome & Parlow, 1974*a,b*), sheep (Grimek & McShan, 1974; Papkoff & Ekblad, 1970) and equine (Landefeld & McShan, 1974) follitropin (follicle-stimulating hormone, FSH) have been isolated and characterized. Further, the primary amino acid sequences of the subunits of human follitropin have been determined (Rathnam & Saxena, 1975; Shome & Parlow, 1974*a,b*). At present, however, no information is available on the subunit structure of bovine follitropin, even though the amino acid sequences of bovine lutropin (luteinizing hormone, LH) and thyrotropin (thyroid-stimulating hormone, TSH) have both been elucidated (Pierce, 1971; Pierce *et al.*, 1971). I have successfully prepared highly purified bovine follitropin, having a biological potency of 160 times that of the reference standard NIH-FSH-S1 (Cheng, 1976); and the presence of subunits in bovine follitropin has been demonstrated (Cheng, 1977). The present paper describes the isolation and characterization of the α - and β -subunits of bovine follitropin.

Materials and Methods

Materials

Purified bovine follitropin (potency: 160 times that of the standard NIH-FSH-S1) was prepared as described previously (Cheng, 1976). Highly purified

bovine lutropin (potency 2.0 times that of the standard NIH-LH-S1) and thyrotropin (potency 30 i.u./mg) and their α - and β -subunits were gifts from Dr. J. G. Pierce, University of California at Los Angeles, Los Angeles, CA, U.S.A. DEAE-cellulose (Cellex-D) was purchased from BioRad Laboratories, Mississauga, Ont., Canada, and cross-linked dextran gel, Sephadex G-100, was from Pharmacia (Canada), Dorval, Quebec, Canada. Propionic acid was obtained from Fisher Scientific Co., Fair Lawn, NJ, U.S.A., and ultrapure urea was from Schwartz-Mann, Orangeburg, NY, U.S.A. All other reagents and chemicals were reagent grade.

Protein determination

Distribution of proteins in eluates after fractionation on ion-exchange column chromatography and gel filtration on Sephadex G-100 was monitored by measuring the A_{278} on a Beckman Instruments Inc. (Palo Alto, CA, U.S.A.) model 25 spectrophotometer.

Dissociation of bovine follitropin

Dissociation of intact follitropin into its α - and β -subunits was accomplished by treating the intact hormone at 1 mg/ml with 1M-propionic acid overnight at 23°C (Laio & Pierce, 1970; Cheng, 1977) followed by freeze-drying.

DEAE-cellulose column chromatography

For separation of the dissociated α - and β -subunits, the freeze-dried material was dissolved at 10mg/ml in 7M-urea solution of 0.04M-Tris/HCl, pH8.2; and the pH of the protein solution was re-adjusted with either 0.1M-HCl or 0.1M-NaOH for chromatography. The DEAE-cellulose column was equilibrated in 0.04M-Tris/HCl buffer at pH8.2, without urea (Cheng, 1976). Under these conditions, the α -subunit was unadsorbed and eluted with the solvent front, whereas the β -subunit and the undissociated follitropin were adsorbed and were eluted together by a 0.25M-NaCl solution in the Tris/HCl buffer. The appropriate fractions were pooled, dialysed against water and freeze-dried.

Gel filtration on Sephadex G-100

The isolated α - and β -subunits of bovine follitropin were finally purified by gel filtration on Sephadex G-100 in 0.5% NH_4HCO_3 buffer at pH8.2. The appropriate fractions of the subunits were pooled and freeze-dried.

Recombination of subunits

For studies on the time course of recombination of the α - and β -subunits of bovine follitropin, 500 μg each of the subunits were incubated in 1ml of 0.012M-sodium glycinate buffer, pH9.5, at 37°C (Laio & Pierce, 1970). At specified time intervals, 10 μl of the incubation mixture containing 10 μg of protein was taken up and diluted immediately to 1ml with ice-cold 0.025M-Tris/HCl buffer, pH7.2, containing 0.1% bovine serum albumin and kept frozen until assayed (Cheng, 1977). The regeneration of follitropin activity was monitored by a specific radioligand-receptor assay (Cheng, 1975).

For studies on recombination of the α - and β -subunits of follitropin, lutropin and thyrotropin, 50 μg of each of the subunits were incubated overnight in 100 μl of 0.012M-sodium glycinate buffer, pH9.5, at 37°C. For testing regeneration of follitropin, lutropin and thyrotropin activities, 10 μl of the incubation mixture was taken up as described above and monitored by specific radioligand-receptor assays; and the remaining 90 μl of the incubation mixture was further characterized by polyacrylamide-gel electrophoresis.

Radioligand-receptor assays

To monitor the regeneration of follitropin, lutropin and thyrotropin activities specific radioligand-receptor assays for these hormones were used as described previously (Cheng, 1975, 1976; Workewych & Cheng, 1978).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis was performed by the method of Davis (1964). Gels (0.4cm $\times$ 6.0cm) of 7.5% (w/v) polyacrylamide were used as described previously (Cheng, 1976). The gels after electrophoresis were stained by 1% (w/v) Amido Black 10B solution in 7% (v/v) acetic acid.

Amino acid analysis

Samples (500 μg each) of purified α - and β -subunits of bovine follitropin were hydrolysed with 6M-HCl in evacuated, sealed tubes at 110°C for 22h. Amino acid analysis were performed with a Beckman model 120c amino acid analyser.

N-Terminal amino acid analysis

The dansyl chloride (5-dimethylaminonaphthalene-1-sulphonyl chloride) method of Gray (1967) was used for the determination of the N-terminal residue. The dansylated protein samples were hydrolysed in 6M-HCl *in vacuo* for 16h at 105°C. The dansylated N-terminal amino acid was identified by comparison with dansylated amino acid standards on two-dimensional t.l.c. on polyamide layers by the methods of Woods & Wang (1967).

Results

Isolation of subunits

The dissociated bovine follitropin, after treatment with 1M-propionic acid, was resolved by DEAE-cellulose into two protein fractions. One fraction was unadsorbed and eluted with the solvent front; the adsorbed fraction was eluted by a 0.25M-NaCl solution of the buffer. Under identical conditions, intact follitropin was adsorbed by DEAE-cellulose and eluted similarly by 0.25M-NaCl solution. Thus the unadsorbed fraction contained the α -subunit and the adsorbed fraction consisted of the β -subunit and the undissociated follitropin.

The fractionated follitropin α - and β -subunits were further purified by gel filtration on Sephadex G-100 as shown in Fig. 1. The unadsorbed fraction after DEAE-cellulose (α -subunit) was eluted as one symmetrical protein peak (Fig. 1a); whereas the adsorbed fraction (β -subunit and undissociated follitropin) was resolved into two protein peaks (Fig. 1b). The elution volume of the larger protein component was observed to be identical with that of the intact follitropin when eluted in the same Sephadex G-100 column. The further retarded smaller protein component (β -subunit) was further purified by rechromatographing once more through the same column of Sephadex G-100. The amounts of

Fig. 1. Gel filtration of the bovine follitropin α - and β -subunit fractions (after DEAE-cellulose) on Sephadex G-100 in 0.5% NH_4HCO_3

Amounts of 13.4mg of the DEAE-cellulose unadsorbed α -fraction (a) and 20.5mg of the adsorbed β -fraction (b) were dissolved separately in 1ml of buffer and applied individually on to the column (1.4cm $\times$ 300cm). Fractions (2ml) were collected at a flow rate of 10ml/h.

Fig. 2. Recombination of purified bovine follitropin α - and β -subunits at different time intervals of incubation
Amounts of 500 μg each of the subunits were incubated at 37°C in 1ml of 0.012M-sodium glycinate buffer, pH 9.5. At different time intervals, 10 μl of the incubation mixture was taken up, and the regeneration of follitropin activity was monitored by a radioligand-receptor assay. Recovery of activity is expressed as a percentage of the native bovine follitropin. ■, ●, Two separate experiments on recombinations of follitropin- α and - β subunits; ▲, follitropin- α ; ○, follitropin- β .

the α - and β -subunits recovered by dissociating 40.0mg of intact bovine follitropin were 12.0 and 10.8mg respectively.

Recombinations of subunits

Fig. 2 depicts the recovery of follitropin activity at different time intervals when a mixture of the isolated α - and β -subunits of bovine follitropin was incubated for recombination of subunits. Follitropin activity was monitored by a specific radioligand-receptor assay. Biological activity was regenerated gradually in a mixture of the α - and β -subunits (Fig. 2) and reached its maximum of approximately 80% of the intact hormone after 10h of incubation at 37°C; incubation of the isolated α - or β -subunit alone did not regenerate any biological activity, being 0.06 and 0.08% of intact follitropin for the α - and β -subunit respectively. These observations indicate a gradual reassociation of the isolated α - and β -subunits of bovine follitropin.

Recombinations of the α - or β -subunit of bovine follitropin with the appropriate β - or α -subunit of bovine lutropin and thyrotropin to regenerate the

Table 1. Recombinations of purified α - and β -subunits of bovine follitropin with the subunits of bovine lutropin and thyrotropin for regeneration of biological activities

Mixtures of 50 μg each of different combinations of purified subunits were incubated in 100 μl of 0.012M-sodium glycinate buffer, pH 9.5, at 37°C for 20h, and biological activities were assessed after appropriate dilutions by specific radioligand-receptor assays for follitropin, lutropin and thyrotropin (Cheng, 1975, 1976; Workewych & Cheng, 1978). Values are means of two separate experiments. Abbreviations used: FSH, follitropin; LH, lutropin; TSH, thyrotropin.

Sample	Dose (μg)	Recovery of biological activity (%)		
		FSH	LH	TSH
FSH	100	100	—	—
FSH- α	50	0.06	—	—
FSH- β	50	0.07	—	—
LH	100	—	100	—
LH- α	50	—	0.05	—
LH- β	50	—	0.10	—
TSH	100	—	—	100
TSH- α	50	—	—	0.04
TSH- β	50	—	—	0.08
FSH- α +FSH- β	50+50	80.40	—	—
FSH- α +LH- β	50+50	0.10	69.80	—
FSH- α +TSH- β	50+50	0.08	—	50.20
FSH- α +LH- α	50+50	0.08	0.10	—
FSH- α +TSH- α	50+50	0.04	—	0.07
FSH- β +LH- α	50+50	76.20	0.12	—
FSH- β +TSH- α	50+50	72.40	—	0.08
FSH- β +LH- β	50+50	0.08	0.07	—
FSH- β +TSH- β	50+50	0.11	—	0.06

Fig. 3. Polyacrylamide-gel electrophoresis on the recombinations of the α - and β -subunit of bovine follitropin with the appropriate β - and α -subunits of bovine lutropin and thyrotropin

Samples (50 μ g each) of the α - and β -subunits were incubated overnight at 37°C in 50 μ l of 0.012M-sodium glycinate buffer, pH9.5, and electrophoresis was performed at 2mA per gel for 1 h. (a) 1, Native follitropin; 2, follitropin- α ; 3, follitropin- β ; 4, follitropin- α +follitropin- β . (b) 1, Follitropin- β ; 2, lutropin- α ; 3, follitropin- β +lutropin- α ; 4, thyrotropin- α ; 5, follitropin- β +thyrotropin- α . (c) 1, Follitropin- α ; 2, lutropin- β ; 3, follitropin- α +lutropin- β ; 4, thyrotropin- β ; 5, follitropin- α +thyrotropin- β .

Fig. 4. Comparison of the elution volume of the α - and β -subunits of bovine follitropin, lutropin and thyrotropin after gel filtration on Sephadex G-100 (1.4 cm $\times$ 300 cm) in 0.5% NH_4HCO_3

●, Native follitropin; □, follitropin- α ; ■, follitropin- β ; △, lutropin- β ; ○, thyrotropin- β . The standards are, from left to right: Blue Dextran, ovalbumin, chymotrypsinogen A, myoglobin and cytochrome c.

specific hormonal activity are summarized in Table 1. The follitropin α -subunit recombined with the β -subunit of lutropin or thyrotropin to regenerate approximately 70% of lutropin or 50% of thyrotropin activity respectively. Similarly, the α -subunit of either lutropin or thyrotropin recombined with follitropin β -subunit to regenerate about 75% of follitropin activity. However, no biological activity was detected in incubation mixtures of either a follitropin α -subunit and another α -subunit or a follitropin β -subunit and another β -subunit (Table 1).

Recombinations of the bovine follitropin α - or β -subunit with the appropriate β - or α -subunit of bovine lutropin and thyrotropin were also demonstrated by polyacrylamide-gel electrophoresis (Fig. 3). After overnight incubation, mixtures of the α -subunit of follitropin, lutropin or thyrotropin and the follitropin β -subunit regenerated protein bands of electrophoretic mobility identical with that of the native follitropin ((Figs. 3a and 3b); whereas the α -subunit of follitropin recombined with the β -subunit of lutropin or thyrotropin to regenerate electro-

Table 2. *Amino acid compositions of the α - and β -subunits of bovine follitropin*

Values were expressed in mol of residues/mol assuming a total of 96 amino acid residues for the α -subunit and 115 residues for the β -subunit, based on the reported sequences of the α -subunit of bovine lutropin or thyrotropin (Pierce *et al.*, 1971) and the β -subunit of human follitropin (Shome & Parlow, 1974b). Residues of half-cystine were determined as *S*-carboxymethylated cysteine of the subunits after derivative formation. Values were 22 h hydrolysates and had not been corrected for the destruction or incomplete liberation of amino acids during hydrolysis.

	α -Subunit			β -Subunit			$\alpha + \beta$ - Subunits	Follitropin (Cheng, 1976)
	Expt. 1	Expt. 2	Mean	Expt. 1	Expt. 2	Mean		
Lysine	10.1	10.4	10.3	6.1	5.7	5.9	16.2	14.2
Histidine	2.9	2.8	2.9	2.8	2.8	2.8	5.7	5.0
Arginine	3.4	3.2	3.3	5.1	4.9	5.0	8.3	8.5
Aspartic acid	6.1	6.3	6.2	10.6	10.8	10.7	16.9	16.6
Threonine	9.3	9.3	9.3	12.9	13.5	13.2	22.5	19.6
Serine	6.2	6.0	6.1	7.4	7.8	7.6	13.7	12.7
Glutamic acid	7.8	7.6	7.7	12.6	12.1	12.4	20.1	19.8
Proline	7.2	6.6	6.9	7.0	7.9	7.5	14.4	10.0
Glycine	3.8	3.5	3.7	6.3	6.3	6.3	10.0	10.3
Alanine	7.0	7.2	7.1	6.4	6.2	6.3	13.4	13.1
Cystine (half)	9.8	10.4	10.1	12.6	11.8	12.2	22.3	17.5
Valine	5.1	5.0	5.1	7.2	7.6	7.4	12.5	12.7
Methionine	4.3	3.8	4.1	0.6	0.6	0.6	4.7	4.0
Isoleucine	2.2	2.1	2.2	5.4	5.6	5.5	7.7	7.2
Leucine	2.3	2.2	2.3	6.4	6.2	6.3	8.6	9.8
Tyrosine	4.4	4.7	4.6	6.2	6.4	6.3	10.9	10.3
Phenylalanine	4.8	4.6	4.7	2.7	2.7	2.7	7.4	6.6

phoretic patterns similar to those of native bovine lutropin and thyrotropin (Fig. 3c). These observations indicate the presence of an interchangeable common α -subunit in bovine follitropin, lutropin and thyrotropin.

Chemical characterization

Fig. 4 shows the elution profiles of the α - and β -subunits of bovine follitropin, comparable with those of the β -subunits of lutropin and thyrotropin, after gel filtration on Sephadex G-100. Under similar experimental conditions, the elution volume of the α -subunits of follitropin, lutropin and thyrotropin was observed to be identical (not shown). It is noted that the follitropin β -subunit was eluted almost identically with the α -subunit; the lutropin β -subunit was eluted slightly ahead and the thyrotropin β -subunit was retarded further behind the α -subunit (Fig. 4). However, the elution volume of any of these subunits was greater than that of the intact hormone on gel filtration on Sephadex G-100.

The amino acid compositions of bovine follitropin and its α - and β -subunits are summarized in Table 2. The α -subunit was low in histidine, arginine, isoleucine and leucine, but relatively high in lysine, threonine, glutamic acid and half-cystine (Table 2). The β -subunit of bovine follitropin contained relatively high amounts of aspartic acid, threonine, glutamic acid and half-cystine and low amounts of histidine and phenylalanine, but probably one methionine residue (Table 2). The *N*-terminal amino

acid residue of the α -subunit was identified to be phenylalanine by the dansyl chloride method. Similarly, the *N*-terminus of the β -subunit was identified to be glycine.

Discussion

The presence of subunit structure in bovine follitropin has been demonstrated after treatment with 1M-propionic acid or 8M-urea followed by gel filtration on Sephadex G-100 and polyacrylamide-gel electrophoresis (Cheng, 1977), and the use of 1M-propionic acid for dissociating bovine follitropin was considered more desirable because much less undissociated follitropin was observed after gel filtration on Sephadex G-100 (Cheng, 1977). In the present report, the α - and β -subunits of bovine follitropin have been successfully isolated by an initial dissociation of the follitropin with 1M-propionic acid followed by redissolving the freeze-dried dissociated material in the presence of 7M-urea for ion-exchange chromatography to separate the subunits. However, a substantial amount of undissociated follitropin was recovered from the β -subunit fraction after gel filtration on Sephadex G-100 (Fig. 1). Most of this undissociated material could not be dissociated into subunits by further treatment with 1M-propionic acid or 8M-urea (K.-W. Cheng, unpublished observation). An amount of 6.0mg of undissociated follitropin was recovered (Fig. 1) upon dissociating 40.0mg of intact follitropin, and the

biological potency of this undissociated material as assessed by radioligand-receptor assay was approximately 10% of that of the intact hormone.

The follitropin activity reconstituted from the subunits regenerated 70–80% of the activity of the intact hormone (Fig. 2 and Table 1). The fact that the α - and β -subunits of follitropin recombined with the respective β - or α -subunit of lutropin and thyrotropin to regenerate 50–80% of the respective hormonal activities (Table 1) suggests that the α -subunit of bovine follitropin is structurally very similar to or identical with the common α -subunit of bovine lutropin and thyrotropin, whereas the β -subunit is the hormone-specific subunit (Pierce, 1971).

The amino acid compositions of the follitropin- α and - β subunits (Table 2) were not highly stoichiometric when compared with that of the intact follitropin (Cheng, 1976). This might be due to microheterogeneity of these glycopolypeptides as displayed upon polyacrylamide-gel electrophoresis (Fig. 3); and heterogeneity of the *N*-terminus has been reported for the α -subunits of sheep and bovine lutropin (Pierce, 1971; Liu *et al.*, 1972a,b) and bovine thyrotropin (Pierce, 1971). However, in the present study on the identification of the *N*-terminus for the follitropin subunits, only one major spot of dansylated amino acid was observed in each case, indicating homogeneity of the polypeptides. The findings that isolated α - and β -subunits, being biologically almost inert at less than 0.1% activity, were able to regenerate upon recombination over 80% of the biological activity (Table 1) and that the regeneration of intact follitropin occurred with a complete disappearance of the α - and β -subunits on recombination (Fig. 3a) are indicative evidences for the purity of the isolated follitropin subunits. The purified α - and β -subunits have also been analysed to be a single protein band by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (K.-W. Cheng, unpublished observation). Furthermore, the amino acid composition of bovine follitropin- α was observed to be almost identical with those of the α -subunits of bovine lutropin and thyrotropin obtained from their amino acid sequences (Laio & Pierce, 1971; Pierce *et al.*, 1971).

The similarity of the amino acid compositions of the α -subunits of bovine follitropin (Table 2), sheep follitropin (Grimek & McShan, 1974) and bovine lutropin and thyrotropin (Pierce *et al.*, 1971; Liu *et al.*, 1972a,b) suggests the possibility that their amino acid sequences may also be very similar, if not identical. The fact that the amino acid composition

of the β -subunit of bovine follitropin was relatively very similar to those of the β -subunits of sheep (Grimek & McShan, 1974) and human follitropin (Shome & Parlow, 1974a,b) indicates that the amino acid sequences of the β -subunits of follitropins of different species may be quite similar. However, the exact chemical relationships between follitropin, lutropin and thyrotropin within and between species will be fully understood only after the complete elucidation of the amino acid sequences of their α - and β -subunits.

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