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Primary structure of the ovine pituitary follitropin α-subunit

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All the tryptic peptides of reduced and aminoethylated α-subunit of ovine pituitary follitropin were isolated. From their composition and partial sequence analysis of the *N*- and *C*-termini of the oxidized protein and reliance on homology with the sequence of lutropin α-subunit, an entire structure for the follitropin α-subunit has been proposed. The structures of the α-subunits from the two ovine hormones are identical.

In common with other glycoprotein hormones, ovine pituitary follitropin (follicle-stimulating hormone, 'FSH') also has two subunits designated α and β (Papkoff & Ekblad, 1974), which are non-identical (Grimek & McShan, 1974; Sairam, 1979*b*). Although follitropin from the ovine species was one of the first glycoprotein hormones to be purified, the structural details of the hormone have not yet been fully understood. However, primary structures for the subunits of follitropin from human (Rathnam & Saxena, 1975; Saxena & Rathnam, 1976), equine (Fujiki *et al.*, 1978) and porcine glands (Closset *et al.*, 1978) have been proposed. It is important to establish the primary structure of the ovine hormone for a better understanding of the structure–function relationships and evolutionary aspects. Accordingly, we have described procedures for the isolation of the highly potent preparation of ovine follitropin (Sairam, 1979*a*) and characterized its subunits (Sairam, 1979*b*). The present series of studies included in this and the following paper (Sairam *et al.*, 1981) describe the primary structure of the α- and β-subunits of this hormone. The data presented herein on the composition and partial sequence of the tryptic and chymotryptic peptides has enabled us to propose the amino acid sequence of the follitropin α-subunit, which is identical with that of ovine lutropin α-subunit.

Materials and methods

Hormone and subunit preparations

Follitropin was isolated from frozen ovine pituitary glands as described previously (Sairam, 1979*a*) and separated into the α- and β-subunits by dissociation in urea followed by ion-exchange chromatography (Sairam, 1979*b*). The chemical and biological characteristics of these preparations have been described in detail (Sairam, 1979*b*).

Reduction and aminoethylation

Approx. 25 mg of the subunit was reduced and alkylated with ethyleneimine (Cole, 1967). The protein (10 mg/ml) was dissolved in 0.5 M-Tris/HCl buffer, pH 8.5, containing 6 M-guanidine hydrochloride (Pierce Chemicals, Rockford, IL, U.S.A.) and 1 mg of disodium EDTA/ml, and saturated with N_2. Dithiothreitol (Calbiochem, Los Angeles, CA, U.S.A.) was added to a 40-fold molar excess over the cystine content. After 4 h of reaction at 37°C, the thiol groups were alkylated by using a 10-fold molar excess (over the dithiothreitol used) of ethyleneimine (Pierce Chemicals). The reagent was added at intervals of 10 min in five portions over a period of 1 h. The reagents were removed by ultrafiltration at 4°C by using a membrane with a nominal molecular-weight cut-off of 1000 (Amicon, Lexington, MA, U.S.A.) followed by dialysis against distilled water for 48 h. The reduced and alkylated product was recovered by freeze-drying. Amino acid analysis of the derivative revealed complete reduction, as indicated by the absence of cystine.

Isolation of peptides

The reduced and alkylated subunits (10 mg/ml) were digested with trypsin (EC 3.4.21.4; Calbiochem, lot no. 387011 B grade) or chymotrypsin (EC 3.4.21.1, Calbiochem, lot no. 400390), an enzyme-to-protein ratio of 1:100 being used. The enzyme was added in two portions 3 h apart. Digestion was allowed to proceed for 6 h at 37°C in 0.25 M-NH_4HCO_3. The reaction was terminated by acidifying with acetic acid. A portion was kept separate to check for the release of free amino acids, if any, by direct amino acid analysis. The rest of the mixture was freeze-dried, redissolved in 0.05 M-NH_4HCO_3 and fractionated on a column (2.5 cm × 90 cm) of Sephadex G-50 (fine grade, Pharmacia) in the same solvent. Thus the peptides were segregated

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into different fractions based on size (see the Results and discussion section). The different freeze-dried fractions were subjected to chromatography and electrophoresis on Whatman 3MM paper, usually on three to four papers each (Sairam & Li, 1977). The dry chromatogram was sprayed with 0.1% ninhydrin in ethanol and dried in a stream of air without heating to reveal the peptide spots. The purple spots as they appeared (usually within 12–24h) were cut out and peptides were eluted with 0.1–0.5M-NH_3 at room temperature. Eluates of similar spots from different chromatograms of the same fraction were pooled and dried in air or freeze-dried.

Homogeneity of the peptides

The homogeneity of the peptides isolated was checked by end-group analysis by the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride method (Gray, 1967) and amino acid analysis. Where necessary, some of the peptides were further purified by chromatography on paper with the butanol/pyridine/acetic acid/water (15:10:3:12, by vol.) system.

Amino acid analysis and end-group determination

Amino acid composition of the peptides was determined after hydrolysis with 6M-HCl (Spackman *et al.*, 1958) by using a Beckman 121 MB amino acid analyser under conditions described previously (Sairam, 1979*b*) or with slight modifications in which the resin W3H was employed. This afforded an improved resolution of the components, including that of hexosamines. *N*-Terminal analysis was performed by the dansyl chloride method (Gray, 1967; Woods & Wang, 1967) and *C*-terminal amino acids were identified by digestion with carboxypeptidases A and/or B (EC 3.4.12.2 and EC 3.4.12.3, Sigma). In the latter case the enzyme digests were directly loaded on to the amino acid analyser for analysis.

Sequence analysis

The amino acid sequence of the peptides was determined by the dansyl–Edman method. All solvents employed were of Sequenal grade (Pierce Chemicals). The procedures for these experiments were slight modifications of the techniques used previously by us (Sairam & Li, 1977). The coupling reaction with phenylisothiocyanate of both peptide and protein was performed with a coupling buffer made up of 10% *N*-ethylmorpholine in water/pyridine (1:1, v/v). The dansyl amino acids were separated by chromatography on polyamide sheets (7.5cm × 7.5cm, Pierce Chemicals) (Woods & Wang, 1967).

Results and discussion

On the basis of amino acid composition and formation of recombinants with hormone-specific β-subunits, it was postulated that the α-subunits of the glycoprotein hormones are probably very similar in structure (Pierce, 1971). This was confirmed by determination of the amino acid sequences of bovine, porcine and human lutropin and thyrotropin (see review by Ward, 1978). Consistent with these observations, the amino acid composition of ovine follitropin α- and lutropin α-(Sairam, 1979*b*) subunits were found to be almost indistinguishable. The α-subunits from two hormones were also interchangeable in recombination studies (Sairam, 1979*b*).

The principal *N*-terminal residues found in oxidized ovine α-subunit were phenylalanine, glycine, aspartic acid and threonine. The most intense spot in the chromatogram of the dansyl derivatives was that of dansylphenylalanine. *N*-Terminal heterogeneity as found in ovine lutropin α-subunit (Sairam *et al.*, 1972*a*) could account for the presence of the other amino acids. The *N*-terminal sequence of the first nine amino acid residues of the oxidized follitropin α-subunit as determined by the dansyl–Edman method was found to be Phe-Pro-Asx-Gly-Phe-Thr-Met(O)-Gly-Glx [Met(O) = methionine sulphoxide], suggesting that peptide chain beginning with phenylalanine was predominant. The presence of *N*-terminal heterogeneity in the α-subunit appears to have no effect on biological activity (Sairam, 1979*b*). Carboxypeptidase digestion was performed to locate the *C*-terminal residues. In common with the lutropin α-subunit (Sairam *et al.*, 1972*a*), the oxidized follitropin α-subunit was easily digested by carboxypeptidase A alone. Kinetic studies were performed by analysing portions of the enzymic digest between 30min and 4h. During the first 30min, serine was detected, followed by progressive increase of lysine, histidine, and tyrosine. At the end of 4h, 1μmol each of serine, lysine, histidine, and 2μmol of tyrosine residues per μmol of the subunit were liberated. These data are identical with those for lutropin α-subunit (Sairam *et al.*, 1972; Liu *et al.*, 1972) and suggest the *C*-terminal sequence to be -Tyr-Tyr-His-Lys-Ser. There was no evidence for *C*-terminal heterogeneity in the follitropin α-subunit. More extensive studies with the ovine lutropin α-subunit have also not indicated heterogeneity at the *C*-terminus. Thus, in α-subunits of both hormones, heterogeneity appears to be confined to the *N*-terminus of the peptide chain.

The tryptic peptide map of oxidized follitropin α-subunit (not shown) revealed only about eight clearly separated spots on paper. The other peptides were not separable because of their size or charge. This result was similar to that obtained with oxidized lutropin α-subunit (Sairam *et al.*, 1972*a*) and hence

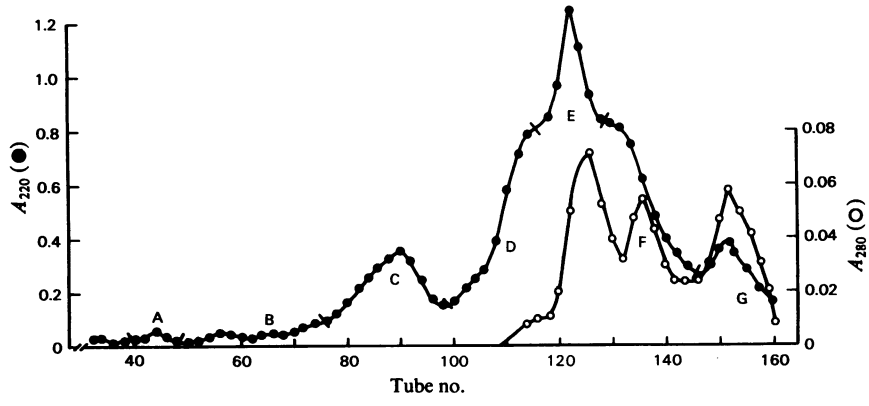

Fig. 1. *Isolation of tryptic peptides of the follitropin α-subunit*

Preliminary separation of tryptic peptides of reduced and *S*-aminoethylated follitropin α-subunit (15mg) was obtained by chromatography on a column (2.5cm × 92cm) of Sephadex G-50 (fine grade) in 0.05M-NH_4HCO_3 at 4°C. Flow rate 16ml/h, 3.2ml per tube. Fractions labelled C-G were used for further separation by paper chromatography [butanol/acetic acid/water ('BAW') system] electrophoresis (pH2.0, 2kV, 1h). The peptide spots were located by staining with ninhydrin and eluted with NH_3. The following peptides were obtained from the different fractions. C: T11, T12; D: T13, T18; E: T1, T3, T4, T5, T8, T9, T14, T15, T16, T17, T21; F: T2, T5, T6, T7, T7a, T8, T10, T17, T19, T20, T22. Fraction G contained salt. For designation of peptide numbers, see Fig. 2.

Table 1. *Amino acid composition (mol of amino acid/mol of peptide) of tryptic peptides of reduced and aminoethylated follitropin α-subunit*

Peptides T2 and T10 are free lysine, peptide T22 is free serine and peptides T13 and T18 contain carbohydrate. *N*-Terminal residues in parentheses were not actually identified. Abbreviation used: Aec, *S*-aminoethylcysteine.

Peptide	Composition	No. of residues	*N*-Terminus
T1	$Aec_{2.3}Thr_{0.7}Glu_{1.8}Pro_{0.7}Gly_{1.0}Met_{0.5}$	8	Thr
T3	$Lys_{1.7}Leu_{1.0}$	3	Lys
T4	$Lys_{1.0}Glu_{0.9}Asp_{1.0}$	3	Glu
T5	$Aec_{0.9}Lys_{0.9}Asp_{1.0}Ser_{0.7}Glu_{0.9}Pro_{2.0}Ala_{1.0}Ile_{0.9}Tyr_{1.7}Phe_{1.0}$	12	Tyr
T6	$Aec_{0.8}Met_{0.7}Gly_{1.0}$	3	Met
T7	$Aec_{0.7}Arg_{1.0}Ser_{0.8}Phe_{1.0}$	4	(Aec)
T7a	$Arg_{1.0}Ser_{0.9}Phe_{0.9}$	3	Phe
T8	$Arg_{1.0}Thr_{0.9}Pro_{1.9}Ala_{2.0}Thr_{0.7}$	7	Ala
T9	$Lys_{1.0}Ser_{1.0}$	2	Ser
T11	$Lys_{1.0}Thr_{0.8}Pro_{1.0}Val_{0.9}Met_{0.7}Leu_{1.0}$	6	Thr
T12	$Lys_{2.0}Thr_{0.8}Pro_{1.0}Val_{0.9}Met_{0.6}Leu_{0.9}$	7	Lys
T13	$Aec_{1.0}Asp_{1.0}Thr_{2.0}Ser_{0.9}Glu_{1.2}Ala_{1.0}Ile_{1.0}$	8	Asp
T14	$Aec_{0.9}Lys_{1.0}Ala_{1.0}Val_{0.9}$	4	(Aec)
T15	$Lys_{1.0}Thr_{0.9}Ala_{1.0}Phe_{1.0}$	4	Ala
T16	$Thr_{0.9}Ala_{0.9}Val_{1.0}Met_{0.7}$	4	Ala
T17	$Arg_{1.2}Asp_{1.1}Gly_{1.0}Val_{1.0}$	4	Gly
T18	$Aec_{0.8}His_{0.7}Asp_{1.0}Thr_{1.2}Ser_{0.3}Glu_{1.8}Ala_{0.3}Val_{0.9}$	7	Val
T19	$Aec_{1.0}His_{0.9}Ser_{0.4}Gly_{0.4}$	2	(His)
T20	$Aec_{1.0}Thr_{0.85}Ser_{1.0}(Gly_{1.8})$	3	(Ser)
T21	$Lys_{1.0}His_{1.0}Tyr_{1.8}$	4	Tyr

was not useful in yielding data on the tryptic peptides. In order to isolate all the tryptic peptides, the reduced and aminoethylated follitropin α-subunit was digested with the enzyme. The digest was separated into (Fig. 1) five fractions on the Sephadex G-50 column. Direct two-dimensional chromatography of each of these fractions yielded 20 peptides plus free lysine and serine. Their compositions are shown in Table 1. Almost all of the peptides were isolated in reasonably good yields

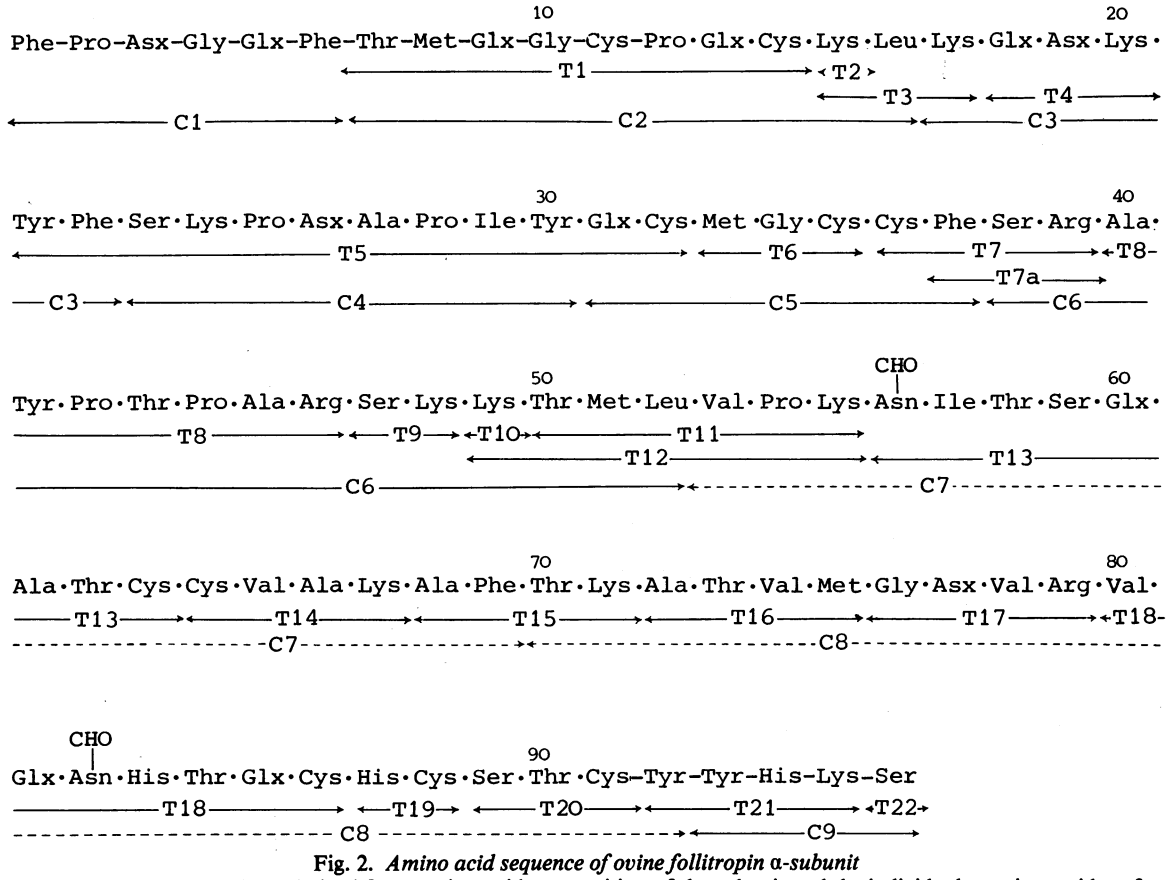

Fig. 2. *Amino acid sequence of ovine follitropin α-subunit*

Data for this proposal are derived from amino acid composition of the subunit and the individual tryptic peptides of the reduced and *S*-aminoethylated α-subunit. Some overlap peptides are derived from chymotrypsin digests. The broken lines for peptides C7 and C8 indicate that these peptides were not isolated. Residues sequenced have been shown as Phe-Pro-Asx- and those placed by amino acid composition as Tyr·Phe·Ser·Lys-... The sequence proposed and the point of attachment of carbohydrate moieties (CHO) is based on homology with the ovine lutropin α-subunit (Sairam *et al.*, 1972*a*; Liu *et al.*, 1972).

(20–40%). Even the two glycopeptides, T13 and T18, were clearly separated. The compositions of all these peptides indicated that they were obtained in a homogeneous state. This was confirmed by end-group analysis. The total number of amino acid residues in these peptides is identical with those in the tryptic peptides of ovine lutropin α-subunit predictable from the reported structure (Sairam *et al.*, 1972*a*, Liu *et al.*, 1972).

From the chymotryptic peptide map of 5 mg of reduced and alkylated ovine follitropin α-subunit, seven peptides whose amino acid composition and end groups could be unequivocally determined were isolated (Table 2). The two chymotryptic glycopeptides, that were in this instance larger than the tryptic glycopeptides (T13 and T18) (Table 1 and Fig. 2) and expected to account for 40 amino acid residues in the subunit, could not be obtained in a pure form. The probable amino acid composition of the glycopeptides was deduced by an inspection of the amino acid sequence of the ovine lutropin α-subunit and the known specificity of chymotrypsin (Sairam *et al.*, 1972*a*; Liu *et al.*, 1972). These peptides being large and carrying carbohydrate usually do not separate during high-voltage electrophoresis at pH 2.0.

From these partial analytical data, it is postulated that the primary structure of ovine follitropin α-subunit is identical (Fig. 2) with that of ovine lutropin α-subunit (Sairam *et al.*, 1972*a*; Liu *et al.*, 1972). It should be noted that the positionings of residues 88 and 89 in the proposal for lutropin α-subunit sequence (Sairam *et al.*, 1972*a*; Liu *et al.*, 1972) are different. Liu *et al.* (1972) reported it as being Cys-Ser, whereas Sairam *et al.*, (1972*a*) reported Ser-Cys. In ovine follitropin α-subunit, the composition of the tryptic peptides T19 and T20 (Table 1), which are not completely homogeneous,

Table 2. *Chymotryptic peptides of reduced and aminoethylated α-subunit (mol of amino acid/mol of peptide)*
Peptides C7 and C8 were complex and both contained carbohydrate. They were estimated to contain 17 and 23 residues respectively (Sairam *et al.*, 1972*a*). Hence they were not separable under the conditions employed. The peptides were separated by direct two-dimensional chromatography–electrophoresis of the digest on paper (2.5 mg per sheet). Abbreviation used: Aec, *S*-aminoethylcysteine.

Peptide no.	Composition	No. of residues	*N*-terminus
C1	$Asx_{0.9}Glx_{1.0}Pro_{0.9}Gly_{1.0}Phe_{1.7}$	6	Phe
C2	$Aec_{1.8}Lys_{1.0}Thr_{0.9}Glx_{2.0}Pro_{0.9}Gly_{1.0}Met_{0.7}Leu_{1.0}$	10	Thr
C3	$Lys_{2.0}Asx_{1.0}Glx_{1.0}Tyr_{0.8}Phe_{0.8}$	6	Lys
C4	$Lys_{1.0}Asx_{0.9}Ser_{0.8}Pro_{2.0}Ala_{1.0}Ile_{0.9}Tyr_{0.8}$	8	Ser
C5	$Aec_{2.5}Glx_{1.0}Gly_{1.0}Met_{0.7}Phe_{0.9}$	7	Glu
C6	$Lys_{1.8}Arg_{2.0}Thr_{1.6}Ser_{1.5}Pro_{1.8}Ala_{2.0}Met_{0.6}Leu_{0.9}Tyr_{0.8}$	15	Ser
C9	$Lys_{1.0}His_{0.9}Ser_{0.8}Tyr_{0.5}$	4	Tyr

would tend to suggest that the sequence of these residues may be Cys-Ser. From known specificity of trypsin, cleavage of the sequence Glx^{85}-Aec^{86}-His^{87}-Aec^{88}-Ser^{89}-Thr^{90}-Aec^{91}- (Aec = aminoethylcysteine) might be expected to produce the peptides His^{87}-Aec^{88}- and Ser^{89}-Thr^{90}-Aec^{91}. The composition of peptides T19 and T20 (with the exception of the glycine residue found) would favour this assignment. On the basis of evidence from other species, namely human lutropin, thyrotropin and follitropin, bovine and porcine lutropin and thyrotropin, in which detailed studies have shown identity, we felt that it was unnecessary to determine the sequence of all the isolated tryptic and chymotryptic peptides. This assumption is not unreasonable, and in fact the analogy was used by us to determine the structure of human lutropin α-subunit for the first time (Sairam *et al.*, 1972*b*).

The composition of peptide T16 (Table 1) and its placement in the follitropin α-sequence (Fig. 2), based on homology with the experimentally determined sequence of ovine lutropin (Sairam *et al.*, 1972*a*; Liu *et al.*, 1972), reveals that an unusual cleavage of the peptide bond involving methionine-75 must have occurred. This cannot be explained at present by the known specificity of trypsin. However, it may be noted that, in our previous studies with ovine lutropin α-subunit, we also observed a cleavage at asparagine-77 by trypsin (Sairam *et al.*, 1972*a*) which is unusual.

Although the structure of the polypeptide moieties of the α-subunits of ovine lutropin and follitropin appear to be identical, their carbohydrate moieties could be different, as shown by the composition of the different sugar residues (Sairam, 1979*b*). The most notable difference is the presence of sialic acid in the follitropin α-subunit (Sairam, 1979*b*; Grimek & McShan, 1974), which is absent in the ovine lutropin itself. The consequences, if any, of this and other small but perhaps significant differences noted earlier (Sairam, 1979*b*) in relation to biological activity of the hormones, are not fully understood.

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